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Isolation and Characterization of Acidocin A and Cloning of the Bacteriocin Gene from *Lactobacillus acidophilus*

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Acidocin A, a bacteriocin produced by *Lactobacillus acidophilus* TK9201, is active against closely related lactic acid bacteria and food-borne pathogens including *Listeria monocytogenes*. The bacteriocin was purified to homogeneity by ammonium sulfate precipitation and sequential ion-exchange and reversed-phase chromatographies. The molecular mass was determined by high-performance liquid chromatography gel filtration to be 6,500 Da. The sequence of the first 16 amino acids of the N terminus was determined, and oligonucleotide probes based on this sequence were constructed to detect the acidocin A structural gene *acdA*. The probes hybridized to the 4.5-kb *Eco*RI fragment of a 45-kb plasmid, pLA9201, present in *L. acidophilus* TK9201, and the hybridizing region was further localized to the 0.9-kb *Kpn*I-*Xba*I fragment. Analysis of the nucleotide sequence of this fragment revealed that acidocin A was synthesized as an 81-amino-acid precursor including a 23-amino-acid N-terminal extension. An additional open reading frame (ORF2) encoding a 55-amino-acid polypeptide was found downstream of and in the same operon as *acdA*. Transformants containing this ORF2 became resistant to acidocin A, suggesting that ORF2 encodes an immunity function for acidocin A. The 7.2-kb *Sac*I-*Xba*I fragment containing the upstream region of *acdA* of pLA9201 was necessary for acidocin A expression in the acidocin A-deficient mutant, *L. acidophilus* TK9201-1, and other *Lactobacillus* strains.

Lactic acid bacteria (LAB) are important in food fermentation because they are used as starter cultures in most cases and also because they have antimicrobial activity which can be attributed to the production of bacteriocins (23). Bacteriocins are peptides or proteins that show a bactericidal mode of action against bacteria that are usually closely related to the producer culture (45). These compounds are of great interest to the food fermentation industry because they may inhibit the growth of undesirable microorganisms that cause food spoilage and that are pathogenic during food processing and food fermentation. In fact, it has been shown that bacteriocins of LAB are able to inhibit the growth of a food-borne pathogen, *Listeria monocytogenes*, which has recently received increased attention (33, 41, 52). Therefore, investigations of bacteriocin in LAB may offer some potential applicability in food preservation.

On the basis of the protein structure, the LAB bacteriocins consitute a heterogeneous group of small peptides, high-molecular-weight proteins, or protein complexes. Most bacteriocins produced by LAB appear to be small peptides. According to the recent classification for the LAB bacteriocins described by Klaenhammer (23), numerous bacteriocins from LAB would belong to either class I or class II. The class I group contains the so-called lantibiotics (42), which have received increased attention in the last few years. Lantibiotics are small ribosomally synthesized polypeptides containing modified amino acids such as lanthionine and 3-methyl-lanthionine (18). The most prominent lantibiotics is nisin (16), although the LAB lantibiotics recently have been isolated and characterized (10, 28, 35, 44). The class II LAB bacteriocins are usually small, hydrophobic or heat-stable peptides and do not contain unusual amino acids such as lanthionine. The peptide bacteriocins are ribosomally synthesized as precursors with an *N*-terminal extension which is posttranslationally removed behind a conserved Gly-Gly motif. To date, many bacteriocins belonging to this class have been identified and characterized biochemically as well as genetically, such as curvacin A (47); lactacin F (31); lactococcin A, B, and M (15, 43, 49, 50); leucocin A-UAL187 (11); plantaricin A (34); pediocin PA-1 and AcH (27, 29); sakacin A and 674 (13, 14); and carnobacteriocin BM1 and B2 (36).

Within the genus *Lactobacillus*, *Lactobacillus acidophilus* has been especially known to display antimicrobial activity against other LAB (19, 48, 51). Barefoot and Klaenhammer found that a majority (63%) of the *L. acidophilus* strains which they examined produced bacteriocins (2). Nevertheless, little is known about the molecular structure and the genetic determinants of these active compounds. Only lactacin F produced by *Lactobacillus johnsonii* VPI11088 (classified previously as *L. acidophilus* 11088) has been studied at the molecular level so far (1, 9). We found a bacteriocin, termed acidocin A, produced by *L. acidophilus* TK9201, which is a starter organism for the production of fermented milk. Here, we describe the isolation and characterization of acidocin A from *L. acidophilus* TK9201. Acidocin A inhibits growth of selected species of LAB, food spoilage bacteria, and food-borne pathogens, including *Listeria monocytogenes*, and has some features in common with a family of class II LAB bacteriocins. The cloning and nucleotide sequencing of the DNA region containing *acdA*, the gene coding for acidocin A, are also described.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. *Lactobacillus* strains were grown in MRS broth (Difco Laboratories, Detroit, Mich.) or MRS agar (1.5%) at 37°C. For production studies at controlled pH, a 5% (vol/vol) transfer from an overnight culture of *L. acidophilus* TK9201 was inoculated into a jar fermentor (2,000-ml working volume of MRS broth) connected to an automatic pH controller. The initial pH was adjusted with concentrated hydrochloric acid to the specified pH, and during fermentation the pH was controlled by the addition of an ammonium hydroxide solution (15%). *Escherichia coli* was grown in LB broth (Difco Lab-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
L. acidophilus		
TK9201	Acd^+ Acd^r, pLA9201	This study
TK9201-1	Acd^- Acd^s, plasmid-free derivative of TK9201	This study
TK9201-1T	Acd^+ Acd^r, TK9201-1 transformed with pESX72	This study
TK1-5	Acd^- Acd^s, pLA106	21
TK1-5T	Acd^+ Acd^r, TK1-5 transformed with pESX72	This study
JCM 2010	Acd^- Acd^s, acidocin A-sensitive indicator	JCM
L. casei		
TK9008	Acd^- Acd^s	21
TK9008T	Acd^+ Acd^r, TK9008 transformed with pESX72	This study
E. coli JM109	*recA1 endA1 gyrA96 thi-1 hsdR17 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q} ZΔM15]	53
Plasmids		
pBluescript IISK+	2.9 kb	Stratagene
charomid 9-36	36 kb	38
pLA9201	45 kb	This study
pULA105E	7.8 kb, Em^r	21
pSEE45	7.4 kb, pBluescript II SK+ carrying 4.5-kb *Eco*RI fragment of pLA9201	This study
pSKX09	3.8 kb, pBluescript II SK+ carrying 0.9-kb *Kpn*I-*Xba*I fragment of pSEE45	This study
pEKX09	8.7 kb, pULA105E carrying 0.9-kb *Kpn*I-*Xba*I fragment of pSEE45	This study
pCB145	17.5 kb, charomid 9-36 carrying 14.5-kb *Bam*HI fragment of pLA9201	This study
pESX72	15.0 kb, pULA105E carrying 7.2-kb *Sac*I-*Xba*I fragment of pLA9201	This study

oratories) with shaking or LB agar (1.5%) at 37°C. When needed, the following antibiotics were used at the concentrations indicated: ampicillin, 50 μg/ml; erythromycin, 200 μg/ml for *E. coli*; and erythromycin, 2.5 μg/ml for *Lactobacillus* strains.

Bacteriocin detection and assay. Bacteriocin production by *L. acidophilus* TK9201 was detected by the direct method of Tagg et al. (45). An overnight culture of strain TK9201 was spotted onto MRS agar and incubated for 16 h to allow colonies to develop. Overlay agar was prepared by mixture with cells of the indicator organism and poured onto the plate. Colonies of strain TK9201 were checked for clear zones around them after incubation.

Acidocin A activity was assayed by the agar-well diffusion method of Tagg and McGiven (46), with some modifications. Portions (100 μl) of serial dilutions of culture filtrates of strain TK9201 were added to wells (1 cm in diameter) cut into the plate, which was inoculated with sensitive indicator cells of *L. acidophilus* JCM 2010 (obtained from the Japan Collection of Microorganisms, Saitama, Japan), and the plate was incubated. The highest dilution that produced a definite zone of growth inhibition of the indicator lawn was defined as 1 arbitrary unit of bacteriocin activity per ml (AU/ml).

Purification. After a 2-liter culture of *L. acidophilus* TK9201 was grown for 12 h at a constant pH of 5.0, cells were removed by centrifugation (8,000 × *g* for 30 min). The supernatant fraction was concentrated by ammonium sulfate (400 g/1,000 ml), and the precipitate pelleted by centrifugation was dissolved with a small amount of 10 mM sodium phosphate buffer (pH 6.5) (buffer A) and then dialyzed against the same buffer. The sample was applied to a column (2.5 by 50 cm) of carboxymethyl-cellulose preequilibrated with buffer A. After being washed with buffer A and buffer A containing 0.3 M NaCl (buffer B), the active bacteriocin was eluted with methanol-buffer B (50/50 [vol/vol]). The active fractions were concentrated to half of the original volume by evaporation and applied to a C_{18} Sep-Pak cartridge (Millipore Corp., Milford, Mass.) activated in accordance with manufacturer's specifications. After being washed with methanol–0.1% trifluoroacetic acid (TFA) (15/85 [vol/vol]), the active bacteriocin was eluted with methanol–0.1% TFA (80/20 [vol/vol]). Active fractions thus obtained were concentrated by evaporation and applied to a reversed-phase C_8 column (2.1 by 220 mm, Aquapore RP-300; Applied Biosystems, Inc.) equilibrated with 0.1% TFA in water. Bound activity was eluted with a linear gradient of 0 to 70% acetonitrile containing 0.1% TFA at a flow rate of 0.2 ml/min. Protein concentrations were determined by using the protein assay kit (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as the standard, or by measuring A_{210}.

HPLC gel filtration. The molecular weight of purified acidocin A was estimated by high-performance liquid chromatography (HPLC) gel filtration (TSK gel $G3000PW_{XL}$ [column, 7.8 by 300 mm]; Tosoh, Tokyo, Japan), with acetonitrile–0.1% TFA (45/55 [vol/vol]) at a flow rate of 0.3 ml/min. The molecular weight markers used and their molecular weights were cytochrome *c*, 12,400 (horse heart); aprotinin, 6,500 (bovine lung); α-melanocyte-stimulating hormone, 1,665; and bradykinin, 1,060.

Amino acid composition and sequence analysis. Purified acidocin A was hydrolyzed with 6 N HCl at 110°C for 24, 48, and 72 h. Each hydrolysate was analyzed by using a JEOL JLC-300 amino acid analyzer (Japan Electric Optical Laboratory, Tokyo, Japan).

An N-terminal amino acid sequence of purified acidocin A was determined by using an Applied Biosystems model 477A protein sequencer with an on-line model 120A phenylthiohydantoin amino acid analyzer (7).

DNA isolation, analysis, and manipulations. Plasmid DNA from *Lactobacillus* strains was prepared as described previously (20). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method (4). Restriction endonucleases and DNA-modifying enzymes were used in accordance with the supplier's specifications. Restriction fragments were isolated and purified from 0.7% agarose gels with a Prep-A-Gene kit (Bio-Rad). Transformation of *E. coli* with plasmid DNA was conducted according to the method of Inoue et al. (17). Other recombinant DNA techniques were carried out as described by Sambrook et al. (39).

Nucleic acid hybridization and nucleotide sequencing. On the basis of the amino acid sequence (KTYYGTNGVH) at positions 1 to 10 in the N terminus of purified acidocin A, the following two 14-mer oligonucleotides were constructed with an Applied Biosystems model 381A DNA synthesizer: ON1, 5′-AA(A/G)AC(T/C/A/G)TA(T/C)TA(T/C)GG-3′, and ON2, 5′-AC(T/C/A/G)AA(T/C)GG(T/C/A/G)GT(T/C/A/G)CA-3′. The oligonucleotides were end labeled with [γ-^{32}P]ATP (Amersham Japan, Tokyo, Japan) by using a 5′ terminus labeling system (Pharmacia, Uppsala, Sweden). Plasmid pLA9201 from *L. acidophilus* TK9201 was digested with the various restriction enzymes, separated on 0.7% agarose gels, and transferred to nylon membranes (Hybond N^+; Amersham) according to the manufacturer's instructions. Hybridization was performed as described by Church and Gilbert (6). To produce suitable clones for sequencing, mutant plasmids with deletions of the inserted fragment were prepared with exonuclease III and mung bean nuclease, by using a Kilo-Sequencing kit (Takara Shuzou, Kyoto, Japan). Sequencing of double-stranded DNA was performed directly by the dideoxy chain termination procedure of Sanger et al. (40) on an Applied Biosystems model 370A DNA sequencer, using a *Taq* Dye Deoxy Termination Cycle Sequence kit (Applied Biosystems). The sequence data was analyzed with the GENETYX software programs (SDC Software Development Co., Ltd., Tokyo, Japan).

Expression of *acdA* gene in *Lactobacillus* strains. Plasmid pLA9201 from *L. acidophilus* TK9201 was digested with *Bam*HI and ligated into the *Bam*HI site of charomid 9-36 (38). The ligated DNA was packaged in vitro by using a Gigapack XL kit (Stratagene, La Jolla, Calif.) and allowed to infect the lysogenic strain *E. coli* JM109. Positive clones were identified by colony blot hybridization with the *Kpn*I-*Xba*I fragment, including the *acdA* gene of pSKX09 as a probe. Plasmid pULA105E was used as an *E. coli-Lactobacillus* shuttle vector (21). Transformation of *Lactobacillus* strains by electroporation with a Gene Pulser apparatus (Bio-Rad) was performed as described previously (21). Bacteriocin production and immunity of transformants harboring different plasmids were tested by using the direct method described above.

Nucleotide sequence accession number. The nucleotide sequence data presented in this study will appear in the GSDB, DDBJ, EMBL, and, NCBI nucleotide sequence databases under accession number D37881.

TABLE 2. Inhibitory spectrum of bacteriocin produced by *L. acidophilus* TK9201[a]

Indicator species	No. of strains inhibited/no. tested
L. acidophilus	8/14
L. brevis	1/3
L. casei	2/6
L. fermentum	1/4
L. plantarum	1/5
Pediococcus acidilactici	0/2
Pediococcus pentosaceus	1/3
Pediococcus halophilus	1/2
Streptococcus bovis	1/3
Streptococcus lactis	5/6
Streptococcus thermophilus	2/4
Food spoilage or pathogenic bacterium	
Bacillus subtilis	0/6
Enterococcus faecalis	1/5
Propionibacterium sp.	2/3
Staphylococcus aureus	0/2
Listeria monocytogenes	5/5

[a] Inhibitory activity was measured by the direct method of Tagg et al. (45).

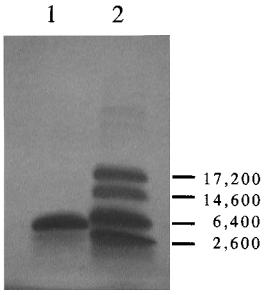

FIG. 1. SDS-PAGE of purified acidocin A. SDS-PAGE was performed in a 15% discontinuous gel by using a Mini-Protean II electrophoresis system (Bio-Rad) according to the method of Laemmli (25). The gel was stained with Coomassie brilliant blue R-250. Lanes: 1, purified acidocin A; 2, molecular size standards (Pharmacia), with sizes given on the right.

RESULTS

Inhibitory spectrum. *L. acidophilus* TK9201 was found to produce a bacteriocin, termed acidocin A. The inhibitory spectrum of acidocin A was investigated by the direct method on several LAB and on various gram-positive bacteria, including pathogenic strains (Table 2). Acidocin A was inhibitory to growth of a variety of closely related bacteria in the genera *Lactobacillus*, *Streptococcus*, and *Pediococcus*. More interestingly, acidocin A was effective against all *Listeria monocytogenes* strains tested. Other gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and gram-negative bacteria (*E. coli* and *Salmonella typhimurium*) were also tested, but no inhibition effect was observed.

Production of acidocin A. Like other bacteriocins found in *L. acidophilus* (3, 30), acidocin A production was affected by the pH of the medium. The optimal broth conditions for the production of acidocin A was evaluated under controlled pH conditions. At pH 5.0, the maximum production of acidocin A (550 AU/ml) was observed at 12 h. No decline in activity was observed over a 20-h period. Little or no activity was detected at pH 6.0, 6.5, or 7.0, although culture growth at each pH was comparable to growth at pH 5.0. Consequently, the culture obtained after the incubation for 12 h at a constant pH of 5.0 was used for acidocin A purification.

Purification of acidocin A. Acidocin A was purified to homogeneity by ammonium sulfate precipitation and sequential cation-exchange and reversed-phase chromatographies. The results are summarized in Table 3. The overall purification procedure resulted in a more than 3,000-fold increase in the specific activity, with a recovery of about 10%. C_8 reversed-phase HPLC on Aquapore RP-300, the final step of the purification, gave a single symmetrical peak of acidocin A activity supperimposable on the major protein peak (data not shown). The homogeneity of purified acidocin A was further established by the appearance on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of a single band at a molecular mass of about 6,400 Da (Fig. 1). The molecular mass of purified acidocin A was estimated to be 6,500 Da by HPLC gel filtration, indicating that acidocin A appears to be a monomeric structure.

Amino acid analysis. The amino acid composition of purified acidocin A after acid hydrolysis is shown in Table 4, assuming the molecular mass of acidocin A to be 6,500. No unusual amino acid residues such as lanthionine were found.

The amino acid sequence analysis of purified acidocin A identified 16 consecutive N-terminal amino acid residues as follows: NH_2-Lys-Thr-Tyr-Tyr-Gly-Thr-Asn-Gly-Val-His-Xaa-Xaa-Lys-Lys-Ser-Leu. Xaa at positions 11 and 12 indicates a blank cycle in which no amino acid derivative was detected.

Identification and cloning of genetic determinant for acidocin A. To detect the acidocin A structural gene, two oligonucleotides (ON1 and ON2) corresponding to the above amino acid sequence were synthesized as probes for the Southern hybridization analysis. Plasmid DNA from *L. acidophilus* TK9201 showed a single hybridizing band of 45 kb, corresponding to plasmid pLA9201. In contrast, the chromosomal DNA from a pLA9201-cured strain, TK9201-1 (acidocin A-deficient mutant), showed no hybridizing signal, indicating that the acidocin A structural gene is located on plasmid pLA9201. To localize the acidocin A structural gene on the plasmid pLA9201, this plasmid was digested with *Eco*RI, ligated to the *Eco*RI site of pBluescript II SK+, and introduced into *E. coli*. Among approximately 400 clones containing inserts, three were found to hybridize to the probes, and these three clones contained a common 4.5-kb *Eco*RI fragment. The recombinant

TABLE 3. Purification of acidocin A

Purification step	Vol (ml)	Total protein (mg)	Total activity (AU)	Sp act (AU/mg)	Yield (%)	Purification (fold)
Culture supernatant	2,000	46,000	1.1×10^6	24	100	1
Ammonium sulfate precipitation	60	4,900	7.2×10^5	1.5×10^2	65	6
Carboxymethyl-cellulose	20	9.2	1.7×10^5	1.8×10^4	15	750
Chromatography						
Sep-Pak cartridge (C_{18} column)	5	1.8	1.2×10^5	6.7×10^4	11	2,790
Aquapore RP-300 (C_8 column)	1	1.5	1.1×10^5	7.3×10^4	10	3,040

TABLE 4. Amino acid composition of the purified acidocin A

Amino acid	No. of residues/molecule from:	
	Amino acid analysis[a]	Nucleotide sequence[b]
Ala	2.11	2
Arg	2.36	2
Asn/Asp	3.35	3
Cys	2.16	2
Gln/Glu	2.20	2
Gly	7.23	7
His	1.81	2
Ile	3.00	3
Leu	7.09	7
Lys	8.85	9
Met	0.31	0
Phe	1.69	2
Pro	1.97	2
Ser	2.13	2
Thr	6.18	6
Trp	1.87	2
Tyr	1.99	2
Val	3.13	3

[a] The values for threonine and serine were calculated by extrapolation of the data determined at 24, 48, and 72 h; the values used for isoleucine and valine were obtained at 72 h. For the analysis of tryptophan, acidocin A was hydrolyzed with 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 24 h. Cysteine was measured as cysteic acid after performic acid oxidation. Other values were averages of hydrolysates at 24, 48, and 72 h. The results are mean values from nine runs for three different bacteriocin preparations.

[b] Deduced from the nucleotide sequence.

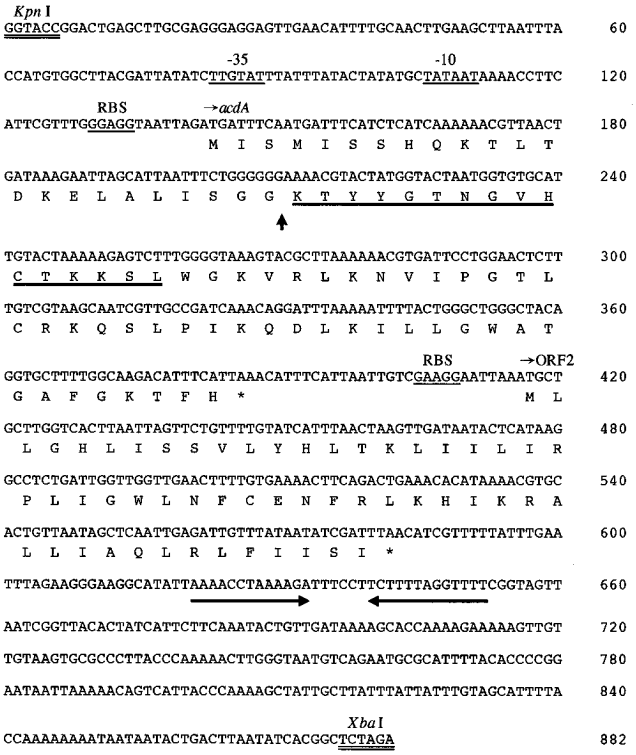

FIG. 3. Nucleotide sequence of 0.9-kb *Kpn*I-*Xba*I fragment from pSKX09. The deduced amino acid sequences (single-letter code) of *acdA* and ORF2 are shown below the nucleotide sequence. The N-terminal amino acid sequence of purified acidocin A is denoted by the thick underline. The vertical arrow between the glycine and lysine at nucleotide position 210 shows the site of cleavage of the N-terminal extension. The putative promoter regions (−35 and −10) and potential ribosome binding sites (RBS) are underlined. The inverted repeat of the terminator is indicated by the horizontal arrows. The termination codons are indicated by asterisks.

plasmid pSEE45 from one of these clones was chosen for further experiments and characterized by restriction enzyme mapping (Fig. 2). Southern blot hybridization analysis showed that the 1.9-kb *Nco*I-*Xba*I and 1.3-kb *Kpn*I-*Nsi*I fragments derived from pSEE45 intensely hybridized to probe ON1. Therefore, the 0.9-kb *Kpn*I-*Xba*I fragment of pSEE45 was subcloned into the *Kpn*I-*Xba*I sites of pBluescript II SK+ to form the recombinant plasmid pSKX09, and a series of deletions of pSKX09 were constructed for nucleotide sequencing.

Nucleotide sequence of acidocin A structural gene *acdA*. The nucleotide sequence of the 0.9-kb *Kpn*I-*Xba*I region of pSKX09 is shown in Fig. 3. Two open reading frames (ORFs) (243 and 165 bp) were observed. The oligonucleotides (ON1 and ON2) used as the probes correspond to the nucleotide sequences at positions 211 to 224 and 226 to 239, respectively. These results suggest that the first ORF, designated *acdA*, is the structural gene encoding acidocin A. Amino acid residues 24 to 39 of the predicted *acdA* protein were homologous to the N-terminal amino acid sequence determined for purified acidocin A, indicating that *acdA* encodes an 81-amino-acid peptide consisting of a 23-amino-acid N-terminal extension and a 58-amino-acid bacteriocin. A probable ribosomal binding site, GGAGG, is located 8 bp upstream from the ATG initiation codon at nucleotide position 142. A putative promoter (coordinates −35 and −10), showing considerable similarity to the typical prokaryotic promoter sequence (24, 32), is found directly upstream of the *acdA* gene.

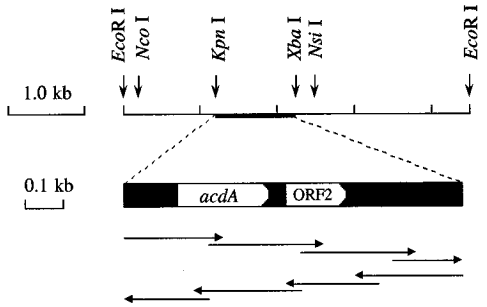

FIG. 2. Linear restriction map of 4.5-kb *Eco*RI fragment of 45-kb plasmid pLA9201 from *L. acidophilus* TK9201. The sequencing strategy and the ORFs are indicated by arrows and boxes, respectively.

The second ORF (ORF2), positioned downstream of *acdA*, was identified from the nucleotide sequence. Taking the ATG at nucleotide position 416 as a translation start codon, this ORF encodes a protein composed of 55 amino acid residues with a molecular mass of 6,480. There is a probable ribosomal binding site (GAAGG) 7 bp upstream from the ATG initiation codon at nucleotide position 416. A 13-bp inverted repeat sequence is present 38 bp downstream of the ORF2 stop codon, probably acting as a transcriptional terminator. No putative promoter and terminator sequences were found between *acdA* and ORF2, indicating that *acdA* and ORF2 may be organized in an operon structure.

Phenotypic expression of *acdA* in *Lactobacillus* strains. The 0.9-kb *Kpn*I-*Xba*I fragment containing the *acdA* gene from pSKX09 was ligated to the *Kpn*I-*Xba*I sites of an *E. coli-Lactobacillus* shuttle vector, pULA105E (21), and the resulting plasmid, pEKX09, was introduced into *L. acidophilus* TK9201-1 (Acd^- Acd^s) by electroporation (21). However,

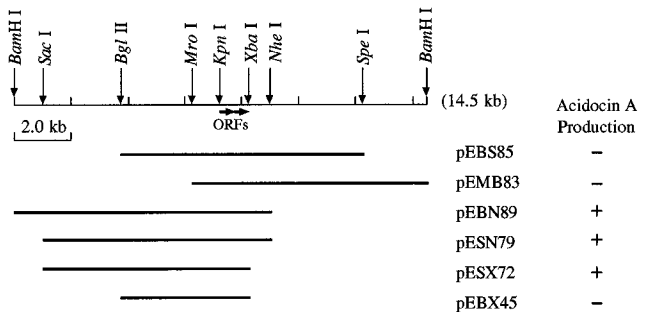

FIG. 4. Linear restriction map of 14.5-kb *Bam*HI fragment of pLA9201 containing the *acdA* gene and its subclones. The lines below the map show subcloned fragments in the indicated plasmids. The acidocin A production phenotypes of *L. acidophilus* TK9201-1 harboring different fragments are indicated on the right.

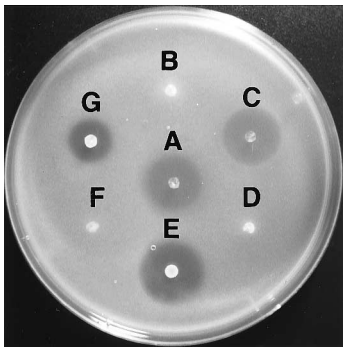

FIG. 5. Growth inhibition of indicator strain *L. acidophilus* JCM 2010 by *L. acidophilus* TK9201 and strains used in transformation experiment by direct method. A, *L. acidophilus* TK9201 (Acd^+ Acd^r); B, *L. acidophilus* TK9201-1 (plasmid pLA9201-cured strain) was an Acd^- Acd^s strain used as the host; C, Acd^+ Acd^r transformant of TK9201-1; D, *L. acidophilus* TK1-5 was an Acd^- Acd^s strain used as the host; E, Acd^+ Acd^r transformant of TK1-5; F, *L. casei* TK9008 was an Acd^- Acd^s strain used as the host; G, Acd^+ Acd^r transformant of TK9008.

transformants harboring pEKX09 failed to produce an active acidocin A, indicating that an additional region is required to obtain acidocin A phenotypic expression. To elucidate the existence of the gene(s) involved in acidocin A expression, cloning of the upstream and downstream regions of the *acdA* gene was done. Plasmid pLA9201 was digested with *Bam*HI, and the 14.5-kb fragment that includes the *acdA* gene was cloned into the *Bam*HI site of the cosmid vector charomid 9-36 (38), yielding the recombinant plasmid pCB145. Specific restriction fragments covering the *Kpn*I-*Xba*I region of pCB145 were cloned on pULA105E, generating various plasmids (pEBS85, pEMB83, pEBN89, pESN79, pESX72, and pEBX45 [Fig. 4]), and their productivity in *L. acidophilus* TK9201-1 was analyzed. As a result, the 7.2-kb *Sac*I-*Xba*I insert of pESX72 was found to be the smallest fragment conferring acidocin A phenotypic expression. These results indicate that this 7.2-kb *Sac*I-*Xba*I fragment contains the genes required to make a functional bacteriocin, as was the case with the bacteriocin operon that has been characterized in LAB (5, 8, 9, 27, 37, 43).

To determine whether this 7.2-kb *Sac*I-*Xba*I fragment of pLA9201 encode all of the genetic information required for acidocin A expression, plasmid pESX72 was introduced into other *Lactobacillus* strains deficient in acidocin A activity. The *Lactobacillus* strains used were *L. acidophilus* TK1-5 (Acd^- Acd^s) and *L. casei* TK9008 (Acd^- Acd^s) (21). Two respective transformants produced the zones of inhibition against the indicator cells (Fig. 5). These transformants also became resistant to acidocin A. Plasmid profiles revealed that pESX72 was retained in the transformants (Fig. 6). These findings clearly indicate that the 7.2-kb *Sac*I-*Xba*I fragment of pLA9201 encodes all the genetic information required for acidocin A expression and host immunity.

DISCUSSION

In this study, acidocin A, a bacteriocin produced by *L. acidophilus* TK9201, has been purified and sequenced. Acidocin A is a 58-residue polypeptide with a molecular mass of 6,503. Acidocin A was completely inactivated by many types of proteolytic enzymes but highly stable during heat treatment at 80°C and boiling for 30 min (data not shown). The production and stability properties in pH-controlled fermentation are similar to those of the bacteriocins, such as lactacins B and F, produced by *L. acidophilus* (3, 30). Amino acid composition analysis of purified acidocin A did not reveal the presence of unusual residues such as lanthionine. Furthermore, nucleotide sequence analysis revealed that acidocin A was synthesized as a precursor. Thus, these biochemical and genetic characteristics indicate that acidocin A produced by *L. acidophilus* TK9201 belongs to the class II LAB bacteriocins (23).

A comparison of the amino acid sequences of the other bacteriocins also confirmed that acidocin A was a class II bacteriocin. As shown in Fig. 7B, the class II bacteriocins contain a positively charged amino acid residue (Lys or Arg) at positions +1 or +2 and in the carboxy terminus an alternate motif of glycines and hydrophobic residues that precede a positively charged terminus (9). The amino acid sequence of acidocin A conforms to these patterns. Furthermore, the amino acid sequences at positions 4 to 11 of acidocin A show similarities to the consensus sequence (Tyr-Gly-Asn-Gly-Val-Xaa-Cys) in the N-terminal region of the anti-*Listeria* bacteriocins, such as carnobacteriocin BM1, curvacin A, leucocin A-UAL187, mesentericin Y105, pediocin PA-1, and sakacin A and 674 (Fig. 7B). This consensus sequence has been suggested to be important for the activity and specificity (against *Listeria monocytogenes*) of this group of bacteriocins (26). Acidocin A also inhibited the growth of all *Listeria monocytogenes* strains tested (Table 2). Therefore, it is conceivable that acidocin A

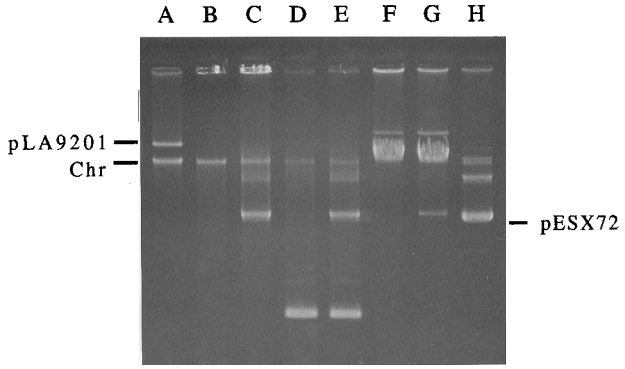

FIG. 6. Analysis of plasmid DNAs of *L. acidophilus* TK9201 and strains used in transformation experiment. Lanes: A, *L. acidophilus* TK9201 (Acd^+ Acd^r); B, TK9201-1 (Acd^- Acd^s); C, TK9201-1T (Acd^+ Acd^r transformant of TK9201-1); D, *L. acidophilus* TK1-5 (Acd^- Acd^s); E, TK1-5T (Acd^+ Acd^r transformant of TK1-5); F, *L. casei* TK9008 (Acd^- Acd^s); G, TK9008T (Acd^+ Acd^r transformant of TK9008); H, plasmid pESX72 from *E. coli* used as the transforming DNA. "Chr" denotes the position of chromosomal DNA.

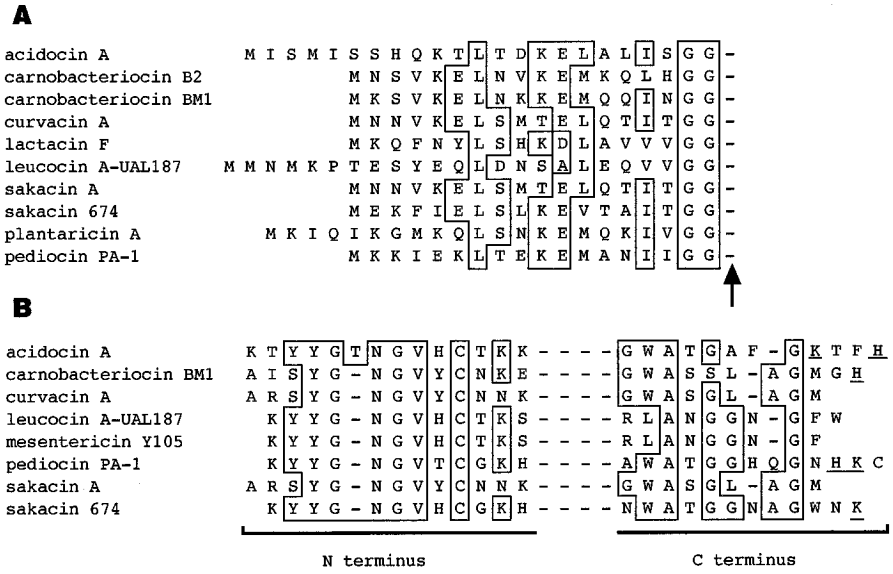

FIG. 7. Amino acid comparison of N-terminal extensions of the class II LAB bacteriocins (A) and anti-*Listeria* bacteriocins (B), carnobacteriocin B2 and BM1 (36), curvacin A (47), lactacin F (31), leucocin A-UAL 187 (11), mesentericin Y105 (12), pediocin PA-1 (27), plantaricin A (8), and sakacin A (13) and 674 (14). The boxed residues are identical for the bacteriocins listed. The underlined amino acids at the C terminus of the mature peptide represent positively charged residues. The arrow indicates the bacteriocin-processing site.

belongs to the anti-*Listeria* family within the class II LAB bacteriocins.

Comparison of the primary structure of the purified peptide with that of the predicted translational product of the acidocin A structural gene (*acdA*) indicates that acidocin A is synthesized as a precursor. The *acdA* gene started with either the initiation ATG codon at nucleotide positions 142 or 151 (Fig. 3). The ATG codon at nucleotide position 142 is the most probable candidate for the initiation codon, because of the putative ribosome binding site, GGAGG, is present at canonical distance (8 bp) from the ATG codon at nucleotide position 142. Taking this ATG as the start codon, the *acdA* gene encodes an 81-amino-acid precursor that undergoes proteolytic cleavage of a 23-amino-acid leader peptide. This leader peptide of *acdA* also has a high degree of homology to those of other class II LAB bacteriocins (Fig. 7A). The Gly-Gly motif, which is typical among the class II LAB bacteriocin precursors previously described, is found at the −1 and −2 positions before the processing site (23). A central region of charged and hydrophilic amino acids and hydrophobic residues at positions −4, −7, and −12 are also presented (9, 36). These similarities found among the leader peptides suggest that a common processing and/or secretion mechanism for the class II LAB bacteriocins could exist.

A pLA9201-cured mutant, TK9201-1, became sensitive to acidocin A, suggesting that pLA9201 carries not only the structural gene for acidocin A but also genetic determinants for host immunity. Analysis of the nucleotide sequence revealed the presence of a second ORF, ORF2, immediately downstream of the *acdA* gene. For other well-characterized bacteriocins, it has been demonstrated that genes involved in immunity functions are closely associated with the bacteriocin structural gene. Therefore, it is possible that the ORF2-encoded protein product may be involved in immunity to acidocin A. However, molecular analyses of ORF2 seem to refute this possibility, because the ORF2 product (55 amino acid residues) is smaller than other proteins responsible for immunity to lactococcins A, B, and M (98, 91, and 154 amino acid residues, respectively) (49, 50), and no amino acid sequence similarity to these protein sequences was found. On the other hand, an acidocin A-sensitive *L. acidophilus* strain, TK9201-1, transformed with the recombinant plasmid pEKX09 carrying the ORF2 gene was resistant to acidocin A (data not shown). This result indicates that ORF2 is the likely candidate to encode an acidocin A immunity function. To clarify the function of this ORF2-encoded protein further investigation is obviously required.

Expression studies revealed that the upstream region of the *acdA* gene is apparently necessary for acidocin A expression in TK9201-1 (Fig. 4). Generally, the structural genes of the class II LAB bacteriocins are located in a gene cluster composed of additional ORFs which are related to activity, to regulatory functions, and to export of bacteriocins (5, 37, 43). The genes from the lactacin F (1) and lactococcin M (49) operons are shown to be required for bacteriocin activities, while the genes from the pediocin PA-1 (27) and plantaricin A (8) operons are involved in the translocation and with the response regulator. Therefore, it seems likely that the upstream region of *acdA* encodes proteins involved in the phenotypic expression of acidocin A.

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