

The Gene Encoding Plantaricin A, a Bacteriocin from *Lactobacillus plantarum* C11, Is Located on the Same Transcription Unit as an *agr*-Like Regulatory System

DZUNG BAO DIEP, LEIV SIGVE HÅVARSTEIN, JON NISSEN-MEYER,† AND INGOLF F. NES*
Laboratory of Microbial Gene Technology, Agricultural University of Norway, N-1432 Ås, Norway

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Purification and amino acid sequencing of plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, revealed that maximum bacteriocin activity is associated with the complementary action of two almost-identical peptides, α and β (J. Nissen-Meyer, A. G. Larsen, K. Sletten, M. Daeschel, and I. F. Nes, *J. Gen. Microbiol.* 139:1973–1978, 1993). A 5-kb chromosomal *Hind*III restriction fragment containing the structural gene of plantaricin A was cloned and sequenced. Only one gene encoding plantaricin A was found. The gene, termed *plnA*, encodes a 48-amino-acid precursor peptide, of which the 22 and 23 C-terminal amino acids correspond to the purified peptides. Northern (RNA) blot analysis demonstrated that a probe complementary to the coding strand of the plantaricin A gene hybridized to a 3.3-kb mRNA transcript. Further analysis of the 3.3-kb transcript demonstrated that it contains three additional open reading frames (*plnB*, *plnC* and *plnD*) downstream of *plnA*. The DNA sequences of *plnB*, *plnC*, and *plnD* revealed that their products closely resemble members of bacterial two-component signal transduction systems. The strongest homology was found to the accessory gene regulatory (*agr*) system, which controls expression of exoproteins during post-exponential growth in *Staphylococcus aureus*. The finding that *plnABCD* are transcribed from a common promoter suggests that the biological role played by the bacteriocin is somehow related to the regulatory function of the two-component system located on the same operon.

A number of lactic acid bacteria produce bacteriocins, which are exoproteinaceous substances exhibiting bacteriocidal activity against related species (19). Such killing may reflect a sophisticated mode of competition among related species sharing the same ecological niche. Bacteriocins from lactic acid bacteria have been grouped into two major classes on the basis of their amino acid composition (19, 29). One class, the lantibiotics, contains the posttranslationally modified amino acids lanthionine and methyllanthionine and their precursors dehydroalanine and dehydrobutyrine (3, 11, 15, 37). The nonlantibiotic class does not contain these modified amino acids. A member of the nonlantibiotic class is plantaricin A, the first bacteriocin which has been purified and characterized from the commercially important species *Lactobacillus plantarum* (30).

The nonlantibiotics represent a relatively heterogeneous group with respect to their primary structure. However, most of them are small peptides (20 to 60 amino acids) that have high isoelectric points (12–14, 24, 27–30, 39, 41–43). The structural genes of nonlantibiotic bacteriocins encode a precursor protein, from which an N-terminal leader peptide is removed to give the mature bacteriocin (14, 24, 27, 39, 41, 43). The leader peptide is cleaved behind two highly conserved glycine residues (14, 24). The similarity found among leader peptides of several nonlantibiotic bacteriocins from lactic acid bacteria indicates that a common processing mechanism for the maturation of these bacteriocins exists.

Recently, cloning and sequencing of the nisin, subtilin, and epidermin operons led to the identification of three open

reading frames (ORFs) which are essential for the production of these lantibiotics (20, 36, 45). The deduced amino acid sequences (NisR, SpaR, and EpiQ, respectively) of the ORFs show significant similarity to those of a family of bacterial signal transduction proteins called response regulators. Response regulators are components of signal transduction pathways in prokaryotes, in which the simplest systems consist of two different proteins: a sensor, often located in the cytoplasmic membrane, and a cytoplasmic response regulator (4, 32, 38). Therefore, these signaling pathways are sometimes called two-component regulatory systems.

In this paper, we report for the first time the cloning, initial characterization, and sequencing of a bacteriocin operon from an *L. plantarum* strain. In addition to the bacteriocin structural gene, the operon contains three ORFs that by amino acid similarity were found to encode a histidine protein kinase and two response regulators. Together, the kinase and the two response regulators make up a complete two-component regulatory system.

MATERIALS AND METHODS

Bacterial strains, cloning vectors, and growth conditions. *L. plantarum* C11, C11D3, and 965 were grown in de Man-Rogosa-Sharpe (MRS) broth (Difco, Detroit, Mich.) without agitation or on MRS plates (1.5% agar) at 30°C (7). Bacteriocin activity was tested by zone inhibition as previously described (26), by using strain *L. plantarum* C11D3 (7) as negative control for plantaricin A production and strain 965 (7) as an indicator. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation. *E. coli* DH5 α cells transformed with pGEM-7Zf(+) and its derivatives were grown in LB broth containing 50 μ g of ampicillin per ml. Standard methods were used for preparing

* Corresponding author. Mailing address: Laboratory of Microbial Gene Technology, Agricultural University, P.O. Box 5051, 1432 Ås, Norway. Phone: 47-64-949471. Fax: 47-64-941465.

† Present address: Department of Biochemistry, University of Oslo, Oslo, Norway.

E. coli K802 for λ infection and strain DH5 α for electroporation transformation (34).

Isolation of plasmids and genomic DNA from *L. plantarum*. Plasmids were isolated from *L. plantarum* by the alkaline lysis method (34). However, 500 U of mutanolysin per ml was used in addition to lysozyme (8 mg/ml), and the final purification step included isopycnic CsCl-ethidium bromide centrifugation. To isolate genomic DNA from *L. plantarum* strains, cells from 100-ml cultures (optical density at 600 nm, 0.6 to 0.8) were pelleted and lysed in 10 ml of TE buffer (200 mM Tris-HCl [pH 8] and 20 mM EDTA) containing 1.5 mg of lysozyme per ml and 125 U of mutanolysin per ml at 37°C for 20 to 30 min. Then, 0.5 ml of 10% (wt/vol) sodium dodecyl sulfate was added, and incubation was continued for 10 min at 62°C. Subsequently, the lysate was extracted several times with Tris-HCl-saturated phenol (pH 7.5), RNase treated, and reextracted with phenol-chloroform before precipitation with ethanol.

Construction of a genomic library, subcloning, and sequencing. Partially *Sau3AI*-digested genomic DNA from *L. plantarum* C11 was size fractionated (34), and DNA fragments ranging from 10 to 20 kb were ligated to dephosphorylated λ -DASH *Bam*HI arms (Stratagene, La Jolla, Calif.). Gigapack II Gold packaging extract (Stratagene) was used to pack the ligated DNA and infect *E. coli* K802 (Clontech, Palo Alto, Calif.). A degenerated probe (termed G4), 5'-TG(T/C)TT(A/G)AT(A/T)GCIGTIGC(A/T)CCCCAT(T/C)TG-3' (see Fig. 1), complementary to nucleotides 638 to 663), deduced from the amino acid sequence of plantaricin A, was used to screen the λ -DASH library. DNA samples obtained from potentially positive plaques were analyzed by Southern hybridization technique, and a positive 5-kb fragment was identified and subcloned into pGEM-7Zf(+) (Promega, Madison, Wis.) for further characterization. All other molecular or microbiological techniques used for screening the library (plating, plaque lifting, DNA isolation, etc.) were carried out according to standard procedures (34). Nested deletions were made by using the Erase-a-Base kit (Promega), and DNA sequencing was performed by using the Sequenase system (U.S. Biochemicals, Cleveland, Ohio) (35). Both strands were sequenced completely. The data base search and DNA sequence alignments were conducted with programs contained within the Sequence Analysis Software Package (version 7.2) licensed from the Genetics Computer Group (University of Wisconsin, Madison) (8).

Southern and Northern analysis. Genomic DNA from *L. plantarum* was digested with the appropriate restriction enzymes, separated on 0.8% agarose gels, and blotted onto a nylon filter (GeneScreen Plus; New England Nuclear) as described by Lillehaug et al. (23). Hybridization was carried out according to the methods of Church and Gilbert (5) and Lillehaug et al. (23). Conditions for hybridization and washing were optimized for each probe. Oligonucleotide probes were end labeled as described by Sambrook et al. (34). For Northern (RNA) analysis, RNA isolated by the method of Igo and Losick (16) was separated on 1.4% agarose in the presence of 2.2 M formaldehyde, blotted onto nylon filters, and hybridized according to the method of Lillehaug et al. (23).

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been assigned EMBL accession number X75323.

RESULTS

Cloning and DNA sequence analysis of the plantaricin A gene. Genomic DNA from strain C11 was analyzed by means of three different restriction enzymes (*Cla*I, *Eco*RV, and *Hind*III) in combination with Southern hybridization using a degenerated oligonucleotide probe (G4). Unique restriction DNA fragments hybridizing to the probe were identified (data not shown). A genomic library was constructed in λ -DASH and screened with the probe G4. Five λ clones were isolated. Digestion of these λ clones with *Hind*III or *Cla*I and subsequent hybridization using probe G4 revealed unique fragments of the expected sizes. One of the clones, termed λ -C1, was further analyzed by restriction enzymes and Southern blotting with probe G4. A 5-kb *Hind*III fragment was identified, isolated, and subcloned into pGEM-7Zf(+).

The nucleotide sequence of the 5-kb *Hind*III fragment was determined in both directions by the dideoxy-chain termination method. An ORF located approximately 1.2 kb downstream from the 5' end of the *Hind*III fragment was identified as the preplantaricin A gene (*plnA*). *plnA* is the first of four consecutive ORFs which are all transcribed in the same direction (Fig. 1). The ORF encoding plantaricin A starts with a Met codon (ATG) directly downstream of a potential ribosome binding site, 5'-GAGGT-3', and encodes a small polypeptide of 48 amino acids. The second ORF probably starts with a Leu codon 190 nucleotides downstream of the stop codon of the *plnA* gene. The putative initiation codon TTG is preceded by a ribosome binding site-like sequence (5'-GAGGT-3'). The second ORF encodes a protein of 442 amino acids. The third ORF most likely begins with a Val codon (GTG) located next to the stop codon of the second ORF. It is preceded by a 5'-GGAGG-3' sequence which probably serves as a ribosomal binding site. The third ORF encodes a polypeptide of 247 amino acids. The fourth ORF probably starts with a Leu codon (TTG) 118 nucleotides downstream of the third ORF and encodes a protein with the same number of amino acids as the protein encoded by the third ORF. The putative ribosome binding site (5'-GGAGG-3') was located upstream of the fourth ORF at an appropriate distance. The three last ORFs were subsequently found to be cotranscribed with *plnA* (see below), and ORFs 2, 3, and 4 were therefore termed *plnB*, *plnC*, and *plnD*, respectively.

Northern blot analysis of the plantaricin A transcript. A synthetic oligonucleotide termed S14 (5'-GCAGTTGCCCC CATCTGCAAAGAATACGCACTACTC-3'), complementary to the coding strand of *plnA* (Fig. 1), was used to determine the size of the plantaricin A transcript by Northern blot analysis. Samples of total RNA isolated from *L. plantarum* C11D3 and 965 were used as negative controls. The Northern blot analyses (Fig. 2) show strong hybridization of the *plnA*-specific probe to a transcript of approximately 3.3 kb. This transcript is not present in the negative controls. The size of the transcript is close to what would be expected if *plnA*, *plnB*, *plnC*, and *plnD* are cotranscribed. To map the 5' and 3' ends of the plantaricin A transcript, Northern blot analysis was carried out with five different oligonucleotides (S3, S7, S8, S9, and S10) with sequences complementary to the coding strand of *plnABCD* (Fig. 1). The oligonucleotides S3, S7, and S9 hybridized to the 3.3-kb plantaricin A transcript. The nucleotide sequences of two of them, S3 and S9, are complementary to the coding regions of *plnC* and *plnD*, respectively, whereas oligonucleotide S7 recognizes the noncoding region between *plnA* and *plnB*. The oligonucleotides S8 and S10, which recognize regions

1 TGCTGTAAAGAAATGGAGTGAAGTAACTAAAATGAAATCCTACAATATGAAATGAAACCGCGATTTTATAAAGCATATCTTTTGGCTAGGCGTGTAGCTG^{S8}
101 **GATTGGTCTT**AGGAGATGTTTATAAAGTACAATACCGCGGTAGCCCGTGATAATGATATTAATGCAATCTATTTATTTGAGAGCCCTTACA

201 AGTAAGAGAATAAATAATATTAACCGTATTTTGGCAATATTTAAATTTACTTAGAGGAAATGATTGAACTTGTTCAGAAAAATAATATACTAA
301 GTACAGACTAATGGAGTATCTTGAATCTTTAGCGGTACACTTAAGTTAATGTTATTTTATCCTCTAAGTATGTTCAAAGTAATCAAGATCTATTC

401 AAAATAGTACTT⁻³⁵⁽¹⁾GACATTCATCAAAATATTGATT⁻¹⁰⁽¹⁾GATGAT⁻³⁵⁽²⁾AGCAATTCATGGTATT⁻¹⁰⁽²⁾CAGTTAAATTTAAAAAATGACGTTAATAGAAAT
501 AATTCCTCCGTACTCAAAAACACATATCTTAAAAGCGAGGTGATTATT^{plnA}GAAAAATCAAATAAAGGTATGAAGCAACTTAGTAATAAGGAAATGCA
M K I Q I K G M K Q L S N K E M Q

601 AAAATAGTAGGTGGAAAAG^{S14}AGTAGCGGTATCTTTGAGATGGGGCACTGCAATTAACACAGTAAAGAACTGTTAAAAAATGGGGATGGTAATG
K I V G G K S S A Y S L Q M G A T A I K Q V K K L F K K W G *
701 ATTTATGAATAACTGTTTTCAAGTTATATTTCTATAGATGGCGTAAAGTGGGCAATTTTAAAGAGTCAACAACCTGAAAAGAGACTATAAAATG
801 ATAATTTGCTGATAAAGTAAACTATAATCATTATTTGAAGAATAGTAGAATGATCGAATAAAAAACTCTTAGAGGTAGTACTGGTGGAAATCA
L V E I S

901 GTATCTCGATAGCGTATTAATCGTTTTTCATTTATCTTGGCATTATCTTGGTATATACTTCACTTTCAGTAATACGAACATTAAGAATCATTTA
I F D S V I Q S F F I Y L G I I L V Y N F I F S N T N I K R I I Y
1001 TTCGTGATTTTACTAGTGTCTCCTTACTAGGAGCTATCTTGGATACACTACATCGTAATTTTGGTGGGCAATTTTAAAGGAAAGTGC
S L I L L L V L S L G A I L G D T T S G L T I L V N T I K E K A A C
1101 CAACCAAAAATAAATATTAATCTAAATGTTCTTAAGTGTGATTCAGTACAGATGTAATTTTGGTGGCTTCTTATTTAAGTAGAGGATTT
Q P K I N Y Y H L N V F L M L I S S Q I V I L A L A S Y I L A S Y R G F L
1201 TGTAATCTACTAGATGCCGATAAAATTTCTGGCTTGTCCGAGTATGGTATTATCTTGGTATTGAAATTTGATGATATTTACGGATTTT
Y I Y L D A D K I S G L S E Y G D L F I G I E I I V M Y I G F L
1301 GGTCTCAATCTGTGATCAAAATGGTAGAGATACACAGGCTTGTGATATAAGTGTGACGAAGAAATAAAGGACTTATCAATATATTGTTA
V F N L V Y K M V R R Y T G S F D I S D D E R I N R H L I T L S
1401 GCTTCTTGGATCAATAGAAATGTTGTTTATTAGCACTTCAAGGTGACAGCTCAACTCAACTTACTGTTGACATTTGTTGATGC
A F L F G S I E M L F I S N K Q G V T A T I Q C T L L T F V I M L G
1501 TTGGCTTATGTTGGCAACATAGAAAAGCATTAGAGTATATGCTTGGCAAAAAGATAGCAGCTGAAAATGCAGAATAAGCAATTAATGATT
G L I S W Q T L E T I R V Y A W Q K K I A A E K L Q N K Q L N D Y
1601 TCTTAAGAGTGTGAGCATCAATATCTAGAAATTAAGAAAATTTAAACATGATATAAGAACCCTAATGCTAGTTAAATCTCAAGATAAATAAGTGA
L K S V E H Q Y L E L R K F K H D Y K N L I A S L N T Q D N I S E
1701 ATTAAGGATTTAGCCGACTATACCCGAGCGGAGAAATTCAGACAAAGCCATAATGACGCTAGCATTGCAAGTGTTCAGCATGGAAGATGAGATA
I K D Y L T D Y T Q S G E F R A S L N D G S I A S V Q H L K N E I L
1801 TGCCGGGTTAGTTGACAGAGTTTTTTATGCGAAAAGTGGGGTTAAGTTGACGATGAAATAGCTAACACTGACTTTATTCTCATGCTGT
R G L V V Q R F F Y A K Q C G V K L T I E I A N T D F I I S H V Y
1901 TACAGTGCCTGACTGATTTATGTAATTTATGGATAATGCCATTGAGCAAGCCCAAAAATGACTGATAAATAGTGACAGTACTTTAATGAAAT
T V A V R I I G N L L D N A I E Q A Q K M T D K I V T V A F N E I
2001 GATAACCCGCTGAAATGCTAGTAACTCTGATTAATGATGATTTAATGACGATCAATTTTGAACCTGGTATTCTCACTAAGGAGTAACTCGT
D N T A E I A I S N P I D S D F N Q H Q I F E T G Y S N R G
2101 CCTTAGGTTGACTAATGTCGGGATCTGTTGACAGCAGAAGGTTTCTATATGGATTTGAAACCAAGAAAATTTGTCACAATCACTGATGAT
L G L T N V R D L V E Q Q K G L F Y M D I E T K K N Y V T M T L I V
2201 TACGGAGATAAATAAGTGTTCCTAATTTATTATTAGAAGATAACGAGGCCCAAGAGTAGATATAAGTATTATAAAGAATAATCAATGATAA
T E D K * V F P I Y L L E D N E A Q R V E Y I S I I K N I I M I K
2301 GAAATGATGACAGTGGTGGTGGCAGCAGAGATTTAACAAGTAAATGAATAATGTTGAATCTAAGAAGCGCTGTTTTCTGGATATGAAA
E Y D M Q L V V A T G D L Q E L M N N V M N S K E G L F F L D M E I
2401 TTGGGAAACAACAGCCGGTGAATTTAGCAGATGAAATTCGGCAGCAGTACCTTGTGCAAAAATGATTTATTAACAACATCAAGAGTATC
G E Q T Q A G L N L A D N A I E Q A Q K M T D K I V T V A F N E I
2501 TTTTGTGACACTGGAGCGCGGATGACCGTTGGATTAATTTGAAAGCAAGCGCTTGCATGATATAAGCAAAAATAGTTAAGGATATGATGCA
F L T L E R R I A P L D Y I L K E Q G L D D I K Q K I V T V A F N E I
2601 ACTCAACTATTCTCAAGCAGAACTGTGAGCATAAGGATATTTAGGTTATAAATCGGAAACGCTTCTTTTCACTGATTAATGATGTTATTA
T Q T I L K T E T V Q H K D I L G Y K I G T R F F S V P I N D V I M
2701 TGCTGAGCACTAATAAGAACGACCGGCTACTTAGGCTGACTCCAAAATAAAGTGCCAGATTTTCCAGTAATTTAAATAGTTTGAANAATAA
L S T N K E R P G S I R L T A K N K V A D F P G N L N S F N E N Y
2801 CTCACAATTTTGGTGTGATAAAGTTCATGGTAAATAGATATTGTTGATAGTTATGATATCAAAAAAAGAGCTTACCATGATAGATAACAT
S Q F F R C D K S S L V N I D Y V D S Y Q K K E L T M I D N I
2901 AAGTGCAGTCTTCGTATAGAAAGTCTCGGAGCTTAAAATAATTAAGAAAGAAATAGGAAACTTATCTTTTATCCAGGCAATGATCTAGTAT
K C S V S Y R K S R E L K K I L K K K *
3001 CTGTTTTGATAGACTGCTTTTTCATGGTAAGACCAAGCAAACTAATTTTGGACATTTTGGAGCAAGAAATGATTTTCCAAATTTATTATAG
L F P I Y L Y E
3101 AGGATAATGCTGAGCAAAAGATAAATTTGTAAGACCGTCAATAATACAAATGATTAATGAGTTTGCATGCACTAGGCTTGCAGGATGATCA
D N A E Q R D N Y C K T V N N T I M I N E F A M E L R V A T D D Q
3201 AAAAATAATCTAGCAGATTTGAATCAACAAGATGGCCTTTCTTTTGGATATGGAATAATGGTAGGACAAACAGACTGGACTTGAATAGCCAGT
K I I L A D L N Q Q Q D G L F F L D M E I G E D K Q T G L E L A S
3301 CGAATTCGGCAAGTACATGGCTAAATAGTTTCTTACACACAGCAGTACGCTTCTTTGTAACCTGGAACCGGATGACCCGTTGGAT
R I R A T P L A R I V F I T T H D E L S F V T I E R R I A P L D Y
3401 ATATTTGAAAGACCAGTCTGCTGACCTAATTCGCAAAAGGATTTAAGGACATCAATGTAGCAGAGAAGCAATTAAGAAAGCAATTAATGTCAGCGCA
I L K D Q S A D L I T Q R I I K D I N V A Q N E L S K K T N S Q R K
3501 AGATGTTTTAACTATAAGTTAGGACCGGATACCTTCTCCTGATGATGATGATTTGTTGAGTACACTAAAACCTGCGTGGGACCGGACAGTAA
D V F N Y K L G T R Y F S L A L D D V I L L S T S K L R P G S V Q
3601 CTCCATGCTAATAAGGTTGCTGAGTTCCAGGAAATTAATGGCTCGAAGAAAATATCCGCAATTTTCCGATAGAGACTCGCTGGTAA
L H A I N K V A E F P G N L N A L E E K Y P Q P F R C D K S S L V N
3701 ATTTAAATCTTCGGAAGTTTACTATAGAAGAAAAGTGTGCTGACGCTGAAATAGCTGTAAGGCTTCTGTTAGAAAAGTGGGAAATGAA
L N H L R S F D A Y K E A A A A L L D G E I R C K K A S F R K A S R E L N
3801 TAAGTTGTGAGACAACTAGTTTTTATAATATTTAGAGCTTCTGCTAGTGTATTGACAGGGCTTTTGTATGAATAGAAATCTGATTATAT
K L L R D N *
3901 AATATAGGCGCTGAAGATAAGTGGAGTAGGCTCTAATTTGATCTTATGAGTATAAATGACTAAAAGTAAACAGTACTCGATGATAAGTATA
S10

FIG. 1. Nucleotide sequence of the DNA adjacent to the plantaricin A structural gene. ORFs *plnA* (precursor plantaricin A gene), *plnB*, *plnC*, and *plnD* are given by one-letter code. The sequence of plantaricin A determined by amino acid sequencing is indicated by italic letters. Possible Shine-Dalgarno sequences (S/D) and potential -10 and -35 promoter regions are underlined. The sequences of the probes used to map the 3.3-kb plantaricin A transcript (S3, S7, S8, S9, S10, and S14) are complementary to the sequences shown in boldface letters. Overlines at the end of *plnD* indicate inverted repeats that potentially constitute a terminator.

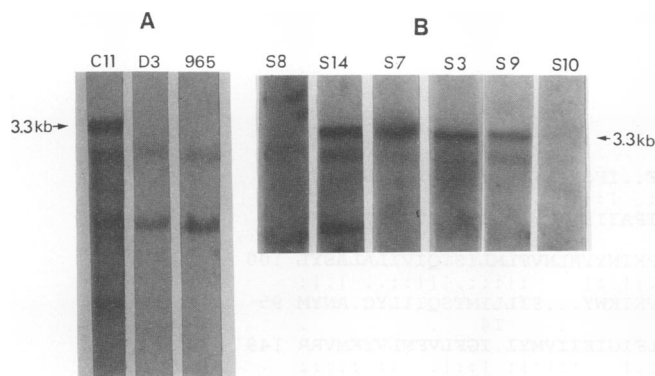


FIG. 2. (A) Northern blot analysis of total RNA isolated from *L. plantarum* C11 (Bac⁺ Imm⁺), C11-D3 (Bac⁻ Imm⁺), and 965 (Bac⁻ Imm⁻) at late exponential phase. Hybridization was performed by using the ³²P-labeled oligonucleotide S14, as described in Materials and Methods. A transcript of 3.3 kb is detected only in the Bac⁺ strain. (B) Northern blot analysis of total RNA isolated from *L. plantarum* C11 at late exponential phase. The genes located on the 3.3-kb transcript were mapped by using different ³²P-labeled oligonucleotide probes (S3, S7, S8, S9, S10, and S14) that hybridize to the coding strand of the *plnA* gene and to sequences upstream and downstream of *plnA* (see Materials and Methods and Fig. 1).

upstream of *plnA* and downstream of *plnD*, respectively, did not hybridize to the 3.3-kb plantaricin A mRNA transcript. Together, these results prove that *plnA*, *plnB*, *plnC*, and *plnD* are located on the same transcription unit.

Genetic localization of the *plnA* operon. To find the genetic location of the *plnA* operon, resident plasmids from *L. plantarum* C11 were isolated, digested with *Eco*RI and *Hind*III, and subsequently analyzed by Southern hybridization using oligonucleotide S14. Genomic DNA from strain C11 and the cloned 5-kb *Hind*III fragment were analyzed in parallel as positive controls. The experiment revealed that the S14 probe gave a positive signal only with genomic DNA and the 5-kb *Hind*III fragment and not with purified plasmids from strain C11, which suggests that the *plnA* operon is located on the bacterial chromosome (data not shown).

DISCUSSION

Purification and amino acid sequencing of plantaricin A have been reported earlier (30). The activity of plantaricin A was associated with two peptides, termed α and β . The β peptide contains an extra alanine residue N terminally; otherwise, the two peptides are identical. The present work demonstrates that a single gene (*plnA*) encodes a plantaricin A precursor protein of 48 amino acids. The α (22-amino-acid) and β (23-amino-acid) peptides are most likely derived from this precursor protein by proteolytic cleavage. By comparison of the amino acid sequences of different prebacteriocins, it was determined that the leader sequences are removed from the prepeptides by cleavage behind the two highly conserved glycine residues (12, 14, 24, 27). The leader sequence of PlnA also contains this consensus sequence of the nonlantibiotic bacteriocins (see the translation product of *plnA* in Fig. 1). However, the sequences of the α and β peptides start 4 and 3 amino acids, respectively, downstream of the two conserved glycine residues. The simplest interpretation of these findings is that PlnA is matured by cleavage behind the two glycines, as found for the other bacteriocins, and that the α and β peptides are the result of

proteolytic degradation during growth and/or purification. However, we and others have previously used the same procedure for the purification of several other lactic acid bacterium bacteriocins (13, 14, 28, 29, 41) without observing proteolytic degradation of the N terminus of the mature bacteriocin. Moreover, the complementary action of the α and β peptides seems to be necessary to obtain maximum bacteriocin activity (30), indicating that this is a specific cleavage of biological significance and not merely the result of nonspecific proteolytic degradation.

Homology searches in the SWISS and PIR data banks showed that PlnB, PlnC, and PlnD belong to a family of prokaryotic regulatory systems termed two-component regulatory systems (4, 32, 38). PlnB, PlnC, and PlnD show highest homology to their counterparts in the *agr* (accessory gene regulatory) two-component regulatory system of *Staphylococcus aureus* (25, 33). PlnB shows 55% similarity and 25% identity to AgrB, the histidine protein kinase of the *agr* system. The size of AgrB is 423 amino acids, which is close to that of PlnB (442 amino acids). Hydrophathy analysis by a method of Kyte and Doolittle (22) predicted that both AgrB and PlnB are transmembrane proteins with six potential membrane-spanning segments located in the N-terminal half of the proteins (Fig. 3A). PlnC and PlnD are highly homologous to each other (58% identity) and showed 27 and 32% identity, respectively, to the *agr* response regulator AgrA (Fig. 3B). Recently, characterization of the gene clusters involved in biosynthesis of the lantibiotics subtilin, epidermin, and nisin led to the identification of ORFs encoding proteins that share sequence homology with bacterial two-component regulatory systems (20, 36, 45). In the case of subtilin and nisin, deletion studies have shown that the regulatory proteins are essential for bacteriocin production (20, 45) but the nature of the extracellular signal(s) that induces synthesis of nisin and subtilin is not known. However, nisin production is stimulated by the addition of sucrose to the medium (9). The synthesis of plantaricin A can be detected very early in the exponential growth phase of *L. plantarum* and seems to proceed at a fairly constant rate until late exponential or early stationary phase. During stationary phase, the number of bacteriocin units per milliliter of medium per bacterium drops sharply (10). We do not know, however, if this change is due to a reduced rate of synthesis or to an increased rate of inactivation of plantaricin A.

The *agr* locus regulates the synthesis of a number of extracellular proteins, many of which play an important role in the pathogenesis of staphylococcal infection (1, 17). The extracellular proteins are subject to growth phase-dependent control (6, 44). Transcription of target genes for alpha toxin, β -hemolysin, δ -lysin, nucleases, and proteases, which are expressed at the end of the exponential phase and the beginning of the stationary phase, is activated by *agr*. However, transcription of target genes expressed during the exponential phase, such as those encoding protein A and coagulase, is repressed by *agr* (17, 44). Analysis of the *agr* locus has revealed that it consists of two divergent transcription units (17, 18, 21). One of these is the *agr* operon, which in addition to the genes encoding AgrA and AgrB, the proteins that make up the two-component regulatory system, also contains two ORFs, *agrC* and *agrD*, of unknown function (Fig. 4). The second transcription unit encodes the 26-residue δ -lysin (*hld*). It has been shown that the gene products of the *agr* operon activate the transcription from their own promoter as well as from the divergent promoter. Thus, activation of transcription of the *agr* operon also leads

A

		T1	
PlnB	4	ISIFDSVIQSFFIYLGIILVYNF . . IFSNTNIKRIIYSLILL . VLSLVG	50
		: : : : : : : : : : : : : : : : : : : : : : : : : : : :	
AgrB	1	MELLSYNFVLFVLTQMILMFTIPAIISGKIKYKLDYFFIIVISTLSLFL	50
		T2	T3
PlnB	51	AILDDTTSLILVLGAIKEKCPKINYYHLNVFLMLISSQIVILALASYL	100
		: . . : : : : : : . : : : : : : : : : . :	
AgrB	51	FKMFDASLI . ILTSFIIIMYFVKIKWY . . SILLIMTSQIILYC . ANYM	95
		T4	
PlnB	101	SRGFLYIYLDADKISGLSEYGDLFIEIIVMYI . IGFLVFNLVYKMR	149
		: : . : : : . : : : : : : : : : : : : : : : :	
AgrB	96	. . . YIVYAYITKISD . . . SIFVIFPSFFVVYVISILFSYIINRVLK	138
		T5	
PlnB	150	YTGSFDISDDERINRHLFIILLA . FFGSIEMLLFISNFQGV TATI . . QLT	196
		: : : : : : : : : : : : : : : : : : : : : : : : : : : :	
AgrB	139	ISTPYLI LNKGFLIVISTILLTLFSLFFYSQINSDEARVIRQYS	183
		T6	
PlnB	197	LLLTFVLMGLISWQ TLETIRVYAWQKKIAAEKLQNKQLNDYLKSEVHQY	246
		: . : : : : : : : : : . : . : . . : : . :	
AgrB	184	LFYWHYHILSILTLYS . . . QFLKEMKYKRNQEETIYYEYTLKIEAIN	229
		T7	
PlnB	247	LELRKFKHDKNLIASLNT . . QDNISEIKDYLTDTYQSGEFRASLNDGS	293
		: : : : . . . : : : : : : : : . .	
AgrB	230	NEMRKRFDYVNIITLSEYIREDDMPGLRDYFNKNIVPMKDNLQMNAIK	279
		★	
PlnB	294	IASVQHLKNEILRGLVQKFFYAKQCGVKLTIEIANTDFILSHGVTVA V	343
		: : : : : : : : : . : : . : : : : : . . : . .	
AgrB	280	LNGIENLKVREIKGLITAKILRAQEMNIPISIEIPDEVSSINLNMIDLSR	329
		T8	
PlnB	344	IIGNLLDNAIEQAQKMTDKIVTVAFNEIDNTAEIAISNPIDSDFNQ . HQI	392
		: . . . : . : : : : . . : . . . : : :	
AgrB	330	SIGIILDNAIEASTEIDDP IIRVAFIESENSVTFIVMNCADDIPRIHEL	379
		★	
PlnB	393	FETGYSTKGSNRGLGLTNVRDLVEQQKGFYMDIETKKNYV	432
		: : : . . : : : : : : 	
AgrB	380	FQESFSTKGEGRGLGLSTLKEIADNADNVLLDTIIENGFF	419
		★ ★	

B

PlnC	V	F	P	I	Y	L	L	E	D	N	E	A	Q	R	V	E	Y	I	S	I	I	K	N	I	I	M	I	K	E	Y	D	M	Q	L	V	V	A	T	G	D	L	Q	E	L	M	N	N	V	M	N	50									
PlnD	L	F	P	I	Y	L	Y	E	D	N	A	E	Q	R	D	N	Y	C	K	T	V	N	N	T	I	M	I	N	E	F	A	M	E	L	R	V	A	T	D	D	Q	K	I	I	L	A	D	L	N	Q	50									
AgrA	-	M	K	I	F	I	C	E	D	D	P	K	Q	R	E	N	M	V	T	I	I	K	N	Y	I	M	I	E	E	K	P	M	E	I	A	L	A	T	D	N	P	Y	E	V	L	E	Q	A	K	N	49									
		★																																																										
PlnC	S	K	E	-	G	L	F	F	L	D	M	E	I	G	E	Q	T	Q	A	G	L	N	L	A	D	E	I	R	Q	Q	L	P	C	A	Q	I	V	F	I	T	T	H	E	E	L	S	F	L	T	L	99									
PlnD	Q	Q	D	-	G	L	F	F	L	D	M	E	I	G	E	D	K	Q	T	G	L	E	L	A	S	R	I	R	A	T	I	P	L	A	K	I	V	F	I	T	T	H	D	E	L	S	F	V	T	L	99									
AgrA	M	N	D	I	G	C	Y	F	L	D	I	Q	L	S	T	D	I	N	-	G	I	K	L	G	S	E	I	R	K	H	D	P	V	G	N	I	I	F	V	T	S	H	S	E	L	T	Y	L	T	F	98									
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PlnC	E	R	R	I	A	P	L	D	Y	I	L	K	E	Q	G	L	D	D	I	K	Q	K	I	V	K	D	I	D	A	T	Q	T	I	L	K	T	E	T	V	Q	H	K	-	D	I	L	G	Y	K	I	148									
PlnD	E	R	R	I	A	P	L	D	Y	I	L	K	D	Q	S	A	D	L	I	T	Q	R	I	I	K	D	I	N	V	A	Q	N	E	L	K	K	T	N	S	Q	R	K	-	D	V	F	N	Y	K	L	148									
AgrA	V	Y	K	V	A	A	M	D	F	I	F	K	D	D	P	A	E	-	L	R	T	R	I	I	D	C	L	E	T	A	H	T	R	L	Q	L	L	S	K	D	N	S	V	E	T	I	E	L	K	R	147									
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PlnC	G	T	R	F	F	S	V	P	I	N	D	V	I	M	L	S	T	N	K	E	R	P	G	S	I	R	L	T	A	K	N	K	V	A	D	F	P	G	N	L	N	S	F	E	N	K	Y	S	Q	F	198									
PlnD	G	T	R	F	F	S	L	A	L	D	D	V	I	L	L	S	T	S	K	L	R	P	G	S	V	Q	L	H	A	I	N	K	V	A	E	F	P	G	N	L	N	A	L	E	E	K	Y	P	Q	F	198									
AgrA	G	S	N	S	V	Y	V	Q	Y	D	D	I	M	F	F	E	S	S	T	-	K	S	H	R	L	I	A	H	L	D	N	R	Q	I	E	F	Y	G	N	L	K	E	L	S	Q	L	D	D	R	F	196									
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PlnC	F	R	C	D	K	S	S	L	V	N	I	D	Y	V	D	S	Y	D	Y	Q	K	K	E	L	T	M	I	D	N	I	K	C	S	V	S	Y	R	K	S	R	E	L	N	K	I	L	K	K	K	247										
PlnD	F	R	C	D	K	S	S	L	V	N	L	N	H	L	R	S	F	D	Y	K	E	K	E	L	L	L	D	G	E	I	R	C	K	A	S	F	R	K	S	R	E	L	N	K	L	L	R	D	N	247										
AgrA	F	R	C	H	N	S	F	V	V	N	R	H	N	I	E	S	I	D	S	K	E	R	I	V	Y	F	K	N	K	E	H	C	Y	A	S	V	R	N	V	K	K	I	-	-	-	-	-	-	-	-	238									

FIG. 3. (A) Alignment of the deduced protein sequences of *L. plantarum* PlnB and *S. aureus* AgrB (EMBL accession no. X52543) histidine protein kinases. The sequences were aligned by using the program BESTFIT (8). Numbers refer to amino acid residues in the proteins indicated on the left. Vertical lines indicate identical residues, while similar residues are indicated by dots. Potential transmembrane sequences (T1 to T6), as predicted by the method of Kyte and Doolittle (22), are shown in boldface letters. Highly conserved residues designated for this protein family are indicated by stars. (B) Amino acid sequence alignment of the PlnC, PlnD, and AgrA (33) response regulator proteins, obtained by using the programs PILEUP and PRETTYPLOT in the Genetics Computer Group sequence analysis software package (8). Boxes indicate identical and similar amino acids. Totally conserved residues in the response regulator family are indicated by stars.

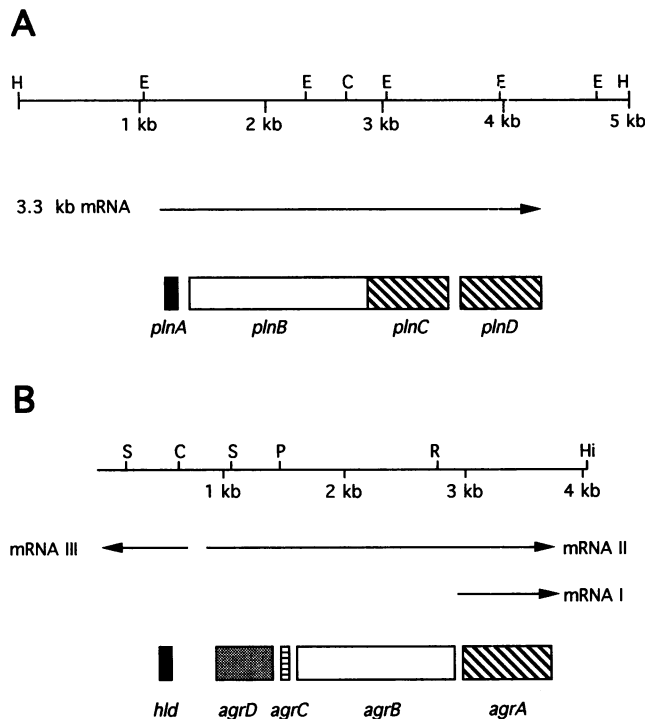


FIG. 4. Comparison of the organization of the plantaricin A operon (A) and the staphylococcal *agr* locus (B) (44). Restriction sites: C, *Cla*I; E, *Eco*RI; H, *Hind*III; Hi, *Hinc*II; P, *Pst*I; R, *Rsa*I; S, *Ssp*I. ORFs *plnA* and *hld* encode the precursor of plantaricin A and δ -lysin, respectively. Both are small peptides capable of lysing sensitive cells. ORFs *plnB* and *agrB* both encode proteins belonging to the histidine protein kinase family. ORFs *plnC*, *plnD*, and *agrA* encode so-called response regulators. ORFs *agrD* and *agrC* encode proteins of unknown function. The arrows indicate known mRNA transcripts.

to increased transcription of the δ -lysin gene (*hld*) (18). Furthermore, Janzon and Arvidson (17) have shown that both the *hld* transcript and its translation product, the δ -lysin, are involved in the *agr*-dependent regulation of synthesis of the extracellular toxins and enzymes mentioned above. In other words, the *hld* transcript and the δ -lysin are part of the *agr* regulatory system. Plantaricin A and the δ -lysin have some very similar properties. Both are small cationic toxins of only 22 to 26 amino acids which are secreted without the typical signal peptide. Analysis by nuclear magnetic resonance and circular dichroism revealed an α -helical configuration of the δ -lysin in both free and lipid-bound states (2, 40). The distribution of the amino acid residues of the δ -lysin is such that when displayed on an Edmundson α -helical wheel, the polar amino acids are found exclusively on one side of the α -helix whereas the nonpolar residues are found on the opposite side (31). Similarly, if displayed on an Edmundson α -helical wheel plantaricin A is also able to form an amphiphilic α -helix (30). In spite of their different origins, many small antimicrobial peptides with a high isoelectric point have in common the capacity to form pores in cytoplasmic membranes (31). The δ -lysin is believed to be a pore-forming toxin (31), and because of the physico-chemical properties of plantaricin A we believe that it kills sensitive bacteria by the same mechanism. On the basis of the genetic organization of the plantaricin A operon and by analogy to the *agr* system (Fig. 4), it might be speculated that

plantaricin A synthesis is regulated by the adjacent two-component regulatory system and/or that plantaricin A forms a part of the regulatory system. Only further investigation, however, will reveal how the two-component regulatory system of the plantaricin A operon functions and what role, if any, the bacteriocin plays in it.

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