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Characterization of a Locus from *Carnobacterium piscicola* LV17B Involved in Bacteriocin Production and Immunity: Evidence for Global Inducer-Mediated Transcriptional Regulation

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Mutational, nucleotide sequence, and transcriptional analyses of a 10-kb fragment (carnobacteriocin locus) from the 61-kb plasmid of *Carnobacterium piscicola* LV17B demonstrated the presence of two gene clusters (*cbnXY* and *cbnSKRTD*) upstream of the previously sequenced carnobacteriocin B2 structural and immunity genes (*cbnB2* and *cbiB2*). Deduced products of *cbnK* and *cbnR* have sequence similarity to proteins of Agr-type two-component signal transduction systems, and those of *cbnT* and *cbnD* have sequence similarity to proteins of signal sequence-independent secretion systems. Deduced products of *cbnX*, *cbnY*, and *cbnS* are class II-type bacteriocin precursors with potential leader peptides containing double-glycine cleavage sites. Genetic analysis indicated that the 10-kb locus contains information required for the production of, and immunity to, the plasmid-encoded carnobacteriocin B2 and the chromosomally encoded carnobacteriocin BM1. In addition, this locus is involved in the production of at least one additional antimicrobial compound and an inducer factor that plays a role in the regulation of carnobacteriocin B2. Transcription analysis indicated that the operons *cbnXY*, *cbnB2-cbiB2*, and *cbnBM1-cbiBM1* (with the latter encoding carnobacteriocin BM1 and its immunity protein on the chromosome) and two small transcripts containing *cbnS* are transcribed only in induced cultures. These transcripts are coregulated and subject to inducer-mediated transcriptional control. Similar regulation of the *cbn* operons is mirrored by the similarity in the nucleotide sequence of their promoter regions, all of which contain two imperfect direct repeats resembling those in Agr-like regulated promoters upstream of the transcription start sites.

Carnobacterium piscicola LV17B is a lactic acid bacterium associated with chill-stored vacuum-packaged meat and produces carnobacteriocins B2 (CB2) and BM1 (CBM1). CB2 and CBM1 are class II pore-forming antimicrobial peptides (bacteriocins) active against many lactic acid bacteria as well as strains of *Enterococcus* species and *Listeria monocytogenes* (2, 21). The potential of these bacteriocins or bacteriocinogenic strains of *C. piscicola* LV17B to eliminate or reduce spoilage and the growth of pathogenic bacteria in foods has stimulated an extensive study of these compounds (19). Phenotypic expression of both carnobacteriocins and immunity to them is dependent on the presence of a 61-kb plasmid (pCP40) that contains the genetic determinants of CB2 precursor (*cbnB2*) and its immunity protein (*cbiB2*) (22). The plasmidless strain *C. piscicola* LV17C, derived from *C. piscicola* LV17B, lacks CB2 and CBM1 production and immunity, despite the presence of the genetic determinants for CBM1 (*cbnBM1*) and its immunity protein (*cbiBM1*) on the chromosome. Cloning of a 10-kb DNA fragment from pCP40 containing *cbnB2* and *cbiB2* into *C. piscicola* LV17C restored production of the plasmid-encoded CB2 and the chromosomally encoded CBM1 and immunity to both bacteriocins (22).

Regulation of carnobacteriocin production by *C. piscicola* LV17B involves peptide-dependent quorum sensing (27). The environmental signal in the culture supernatant of *C. piscicola* LV17B that induces carnobacteriocin production was previously identified as the bacteriocin CB2, indicating autoregulation (27). In addition to induction by CB2, induction of carnobacteriocin production by *C. piscicola* LV17B is also triggered by CB2 recombinant variants purified from *Escherichia coli*, including one with no detectable antimicrobial activity (23). Peptide-based induction of bacteriocin production has also been reported for lactobacilli and lactococci. The best example is the production of the lantibiotic nisin by *Lactococcus lactis*. In addition to its antimicrobial activity, nisin functions as an inducer of its own synthesis through the *nisKR* two-component signal transduction system (16). In contrast, production of a bacteriocin by *Lactobacillus plantarum* C11A and production of the class II bacteriocin sakacin P by *Lactobacillus sake* LTH673 are induced by peptides with no reported antimicrobial activity: plantaricin A, consisting of 26 amino acids, and the induction factor of 19 amino acids, respectively (5, 8). The genes for plantaricin A (*plnA*) and induction factor (*orf-1*) are located upstream of putative *agr*-like two-component regulatory systems (*plnBCD* and *sppKR*, respectively), and they encode the corresponding inducer precursors, each containing a double-glycine leader peptide (7, 12).

The purpose of this study was to gain insight into the genetic requirements for bacteriocin production and immunity and the regulatory mechanism involved in bacteriocin production. To this end, the previously cloned 10-kb DNA fragment was se-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype and property[a]	Reference
Bacteria		
C. piscicola LV17B	Cbn^+ Imm^+, containing pCP40	2
C. piscicola LV17C	*C. piscicola* LV17B lacking pCP40, Cbn^- Imm^-	2
C. divergens LV13	Cbn^- Imm^-	2
Plasmids		
pCaT	8.5-kb cloning vector, Cm^r	14
pCP40	61-kb plasmid conferring Cbn^+ Imm^+ phenotype	2
pLQ24	pCaT containing a 17.2-kb fragment from pCP40	22
pLQ18	pCaT containing a 10-kb fragment from pCP40	22
pLQ18′	pCaT containing the same insert as in pLQ18, but inserted in opposite orientation	This study
pLQ18E	pLQ18 with *cbnB2* and *cbiB2* deleted	22
pLQ18B	pLQ18 with *cbnB2* partly deleted	This study
pLQ11, -12, -15	Deletion derivatives of pLQ18	This study
pLQ18K	pLQ18 with a frameshift mutation in *cbnK* (*Sph*I site)	This study
pLQ18R	pLQ18 with a frameshift mutation in *cbnR* (*Bst*NI site)	This study
pLQ18T	pLQ18 with a frameshift mutation in *cbnT* (*Nhe*I site)	This study
pLQ18D	pLQ18 with a frameshift mutation in *cbnD* (*Ban*II site)	This study

[a] Cbn, carnobacteriocin(s) production; Imm, carnobacteriocin immunity.

quenced and studied by deletion and gene inactivation analyses as well as by transcriptional analysis.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. Bacteria and plasmids are described in Table 1. *Carnobacterium* cultures were grown in APT broth (Difco Laboratories, Inc., Detroit, Mich.) or APT agar plates (1.5% agar) as described previously (2). Chloramphenicol (5 μg/ml) was used for selection and growth of transformants.

Detection of bacteriocin production. Production of bacteriocin on solid medium was detected by deferred inhibition assay (1). Strains to be tested were grown in APT broth (25°C for 18 h) and were spot inoculated onto plates containing 20 ml of APT agar with a multiple replicator (KVL Laboratories, Cambridge, Ontario, Canada). The plates were kept at 25°C for 20 h before being overlaid with 10 ml of soft APT agar (0.75% agar) inoculated (1% [vol/vol]) with a 24-h culture of the sensitive indicator strain *Carnobacterium divergens* LV13 harboring the plasmid pCaT (2). The plates were kept at 25°C for 16 h before the diameter of the zones of inhibition on the lawn of the indicator strain surrounding the producer colony was recorded. Bacteriocin activity in the culture supernatant and titration of the activity of the purified bacteriocins were determined by spot-on-lawn tests (2). Plates containing 20 ml of APT agar were overlaid with 10 ml of soft APT agar inoculated (1% [vol/vol]) with a 24-h culture of *C. divergens* LV13 harboring the plasmid pCaT (2). The culture supernatant to be tested was heat treated (65°C for 15 min), and serial twofold dilutions (in APT broth) were spotted (20 μl) onto the *C. divergens* lawn. The plates were incubated at 25°C for 16 h, and the size of the zones of inhibition was recorded. Results are expressed in arbitrary units (AU) of bacteriocin (1 AU is the amount of bacteriocin required to produce visible clearing on a lawn of the indicator strain). The minimum detection limit is 50 AU/ml.

Bacteriocin sensitivity assay. Sensitivity to CB2 and CBM1 was determined (22) with a microtiter plate reader (THERMOmax Microplate Reader; Molecular Devices Corp., Menlo Park, Calif.). Serial fourfold dilutions of each bacteriocin in APT broth were loaded (200 μl) in microtiter plate wells, inoculated (2.5% [vol/vol]) with a 16-h culture of the strain to be tested, and incubated at 25°C, and absorbance at 650 nm was measured at 30-min intervals in the microtiter plate reader. The data were analyzed with the software SOFTmax, version 2.3 (Molecular Devices Corp.). Maximum growth rates in the presence or absence of purified carnobacteriocins were calculated with six consecutive measurements. Correlation coefficients ranged between 0.98 and 1.00. The immunity of the strains was expressed as a percentage of the growth rate in the presence of bacteriocin relative to the growth rate in the absence of bacteriocin [percent immunity = (growth rate with bacteriocin/growth rate without bacteriocin) × 100]. Bacteriocins used in the tests (CB2, 50 AU/μg; CBM1, 90 AU/μg, determined against *C. divergens* LV13) were purified as previously described (21).

Induction experiments. Induction of nonproducing (Bac^-) cultures of *C. piscicola* LV17B was done as previously described (23). *C. piscicola* LV17B was inoculated below 10^4 CFU per ml to obtain Bac^- cultures. The Bac^- cultures were induced at the time of inoculation by adding heat-treated (65°C, 15 min) culture supernatant (2% [vol/vol]) from selected transformants. After induction, the inhibitory activity of the culture supernatant was evaluated by spot-on-lawn tests when cultures reached an optical density at 600 nm of 0.8.

DNA and RNA manipulations. Isolation of plasmid DNA and transformation of *Carnobacterium* strains were done as previously described (3). RNA for Northern and primer extension analyses was isolated from induced (Bac^+) and uninduced (Bac^-) cultures of *C. piscicola* LV17B by previously described methods (17). Induction was done with heat-treated supernatant (as described above). Primer extension and Northern hybridization analyses with radiolabeled probes prepared by nick translation were done as previously described (17). The primers used for primer extension are listed in Table 2. DNA templates to generate probes radiolabeled by nick translation were amplified by PCR and purified by standard procedures (26). PCR was performed with AmpliTaq DNA polymerase (Perkin-Elmer) as recommended by the supplier. Probe 1 (675 bp) encompassed *cbnBM1* and *cbiBM1* and was amplified from pUC18 containing the *cbnBM1-cbiBM1* insert (21) with forward and reverse primers. Probes 2, 3, and 4 were amplified from pLQ18 (22) with the primers shown in Table 2. Probe 2 (570 bp) encompassed *cbnB2* and *cbiB2*, probe 3 (560 bp) encompassed *cbnS* and the 5′ end of *cbnK*, and probe 4 (310 bp) encompassed *cbnX* and *cbnY*.

Restriction enzymes, Klenow fragment of DNA polymerase I, T4 DNA ligase, and exonuclease III were used as recommended by the supplier (Boehringer Mannheim, Dorval, Québec, Canada). Restriction digestion, agarose gel electrophoresis, exonuclease III treatment, blunt-end creation with Klenow fragment, and DNA ligation were done by standard procedures (26). DNA was sequenced bidirectionally with *Taq* DNA polymerase and fluorescent Dyedeoxy-chain terminators and analyzed on a DNA sequencer (Applied Biosystems 373A; Foster City, Calif.). Sequencing reactions were primed with oligonucleotides synthesized on a DNA synthesizer (Applied Biosystems 391 PCR MATE). Overlapping sequences were assembled and analyzed with DNAStar (Madison, Wis.).

Inactivation of *cbnK*, *cbnR*, *cbnT*, *cbnD*, and *cbnB2*. Inactivation of *cbnK*, *cbnR*, *cbnT*, and *cbnD* was achieved by introducing frameshift mutations at specific *Sph*I, *Bst*NI, *Nhe*I, or *Ban*II sites, respectively (frameshift mutants). The previously described plasmid pLQ18 (22) was partially digested with *Sph*I, *Bst*NI, *Nhe*I, and *Ban*II. The linear plasmids were purified by agarose gel electrophoresis, blunt ended, religated, and electrotransformed into *C. piscicola* LV17C.

TABLE 2. Oligonucleotides used in this study

Primer[a]	Sequence	Description
B2	5′-TACCATAATTTACTCCACCGTG-3′	Complementary to *cbnB2*
S	5′-CAATCTGACTATTTTTACTTCCTC-3′	Complementary to *cbnS*
X	5′-TCCTTCCATCCCCATCCTCC-3′	Complementary to *cbnX*
2	5′-ATGAATAGCGTAAAAGAATTA-3′	Used to amplify probe 2
2′	5′-CGCTCTAGATTAGAAATATATATAAGGAAC-3′	Used to amplify probe 2
3	5′-ATGAAAATAAAAACAATAACCAAG-3′	Used to amplify probe 3
3′	5′-CGTTATCACTAAGACTAATAATTGG-3′	Used to amplify probe 3
4	5′-ATGAAAAGTGTTAAGGAATTAAA-3′	Used to amplify probe 4
4′	5′-CTTCGATCATTTATAGCTTTTTTGAAC-3′	Used to amplify probe 4

[a] Primers B2, S, and X were used in primer extension; all other primers were used in the construction of probes.

Transformants were screened for bacteriocin production. Plasmid DNA from Bac^+ and Bac^- transformants was isolated and screened for the loss of the specific restriction sites. Changes in the nucleotide sequence at the specific positions were confirmed by DNA sequencing. CbnB2 was inactivated by creating a 31-bp deletion in *cbnB2* with exonuclease III after digestion of pLQ18 with *Bam*HI. The deletion replaced the last 12 amino acid residues of CbnB2 with eight different amino acids.

Protein sequence analysis. Protein alignment was performed with GeneWorks 2.3 software (IntelliGenetics, Inc., Mountain View, Calif.). Nonredundant database searching and computations for sequence similarity were performed at the National Center for Biotechnology Information with the BLAST network service.

Nucleotide sequence accession number. The nucleotide sequence reported in this study was submitted to GenBank (Los Alamos, N.Mex.) and was assigned accession no. L47121.

RESULTS

Nucleotide sequence analysis of the locus involved in bacteriocin production and immunity. The previously cloned 10-kb DNA fragment from the plasmid pCP40 was sequenced to gain insight into the genetic requirement for bacteriocin production, immunity, and the cell density-dependent mechanism involved in regulation of carnobacteriocin production. The nucleotide sequence of this fragment and the possible products of identified open reading frames (ORFs) are shown in Fig. 1. ORFs that were transcribed by Northern analyses (see below) or that could be involved in bacteriocin production were labeled as *cbn* genes. Deduced products of the 5′ end of the truncated *orf-1* (187 amino acids) and of *orf-2* (152 amino acids) exhibited significant sequence identity to the transposase IstB from IS*21* (33%) and to the transposase from IS*231*F (36%) (24, 25). The deduced products of *orf-3* (42 amino acids) and *orf-4* (113 amino acid residues) had no obvious sequence similarity to known proteins. The deduced products of *cbnX*, *cbnY*, and *cbnS* (51, 48, and 41 amino acids, respectively) resembled class II bacteriocin-like precursor peptides with potential double-glycine-type leaders (11, 15) (Fig. 2). The product of *cbnS* also exhibited sequence identity (40%) to a polypeptide of 45 amino acid residues of unknown function encoded by *orf-4* in the sakacin A gene cluster of *L. sake* Lb706 (4).

The predicted products of *cbnK* and *cbnR* (CbnK and CbnR), containing 442 and 245 amino acid residues, respectively, exhibited sequence similarity to histidine protein kinases and response regulators of bacterial Agr-type, two-component signal transduction systems (28) (Fig. 1), including products of the genes *sapK* (36%) and *sapR* (57%) of *L. sake* Lb706 (4), *sppK* (20%) and *sppR* (29%) of *L. sake* Lb674 (12), *agrC* (20%) and *agrA* (31%) of *Staphylococcus aureus* (31), and *plnB* (18%) and *plnD*/*C* (28 and 27%, respectively) of *L. plantarum* C11 (7). The partner proteins SapK/R, SppK/R, and PlnB/C/D are believed to be involved in the regulation of class II bacteriocin production, and AgrA/C is believed to be involved in regulation of virulence factors. Predicted products of *cbnT* and *cbnD* (CbnT and CbnD) containing 716 and 455 amino acid residues, respectively, show sequence similarity to ATP-binding cassette (ABC) transporters and accessory proteins of bacterial signal sequence-independent secretion systems (9), including *sppT* (48%) and *sppE* (23%) of *L. sake* Lb674 (12), *sapT* (47%) and *sapE* (23%) of *L. sake* Lb706 (4), *comA* and *comB* (23%) of *Streptococcus pneumoniae* (13), *lcaC* (46%) and *lcaD* (23%) of *Leuconostoc gelidum* UAL187 (30), *lcnC* (44%) and *lcnD* (22%) of *L. lactis* WM4 (29), and *pedD* (43%) of *Pediococcus acidilactici* PAC 1.0 (18). The protein pairs SapT/E, SppT/E, LcaC/D, and LcnC/D are believed to be dedicated processing and secretion systems for class II bacteriocins. PedD is involved in the processing of the double-glycine leader peptide and secretion of pediocin PA-1 (11, 33). ComA and -B are believed to be a dedicated processing and secretion system for a heptadecapeptide pheromone that induces competence in *Streptococcus pneumoniae* (10).

Deletion analysis and inactivation of *cbnK*, *cbnR*, *cbnT*, and *cbnD*. To delineate the region required for a Bac^+ Imm^+ phenotype and to investigate the possible involvement of some of the genes located in the sequenced fragment in bacteriocin production, *C. piscicola* LV17C was transformed with several derivatives of pLQ18. These derivatives were plasmids with either deletion or frameshift mutations in *cbnK*, *cbnR*, *cbnT*, or *cbnD*. The plasmid constructs and the corresponding phenotypes of the transformants are summarized in Fig. 3. The zones of inhibition produced by the transformants against the sensitive indicator *C. divergens* LV13 in the deferred assay are shown in Fig. 4. As expected, transformants containing plasmid pLQ18′, pLQ18, or pLQ24 with the complete gene cluster (Table 1) produced similar zones of inhibition of *C. divergens* (Fig. 4; cultures C, D, and E), while those containing pCaT did not (Fig. 4; cultures M and N). When a frameshift mutation was introduced into *cbnB2* (pLQ18B) or when *cbnB2* and *cbiB2* were deleted (pLQ18E), there was a slight but repeatable decrease in bacteriocin production (Fig. 4; cultures A and B, respectively). Retention of bacteriocin production by these clones is consistent with the known production of the chromosomally encoded bacteriocin CBM1 (22). However, deletion of the genes *cbnT*, *cbnD*, and *orf-4* in pLQ12, besides those genes deleted in pLQ18E (*cbnB2* and *cbiB2*), resulted in complete loss of bacteriocin production (Fig. 4; culture K). This result suggests that at least one or more genes of this region (*cbnT*, *cbnD*, and *orf-4*) are necessary for the production of CBM1. The lack of bacteriocin production of the transformant containing pLQ11 (Fig. 4; culture L) is consistent with this observation. Deletion of a fragment spanning the 3′ end of *orf-1* and the 5′ end of *cbnR* (pLQ15, Fig. 3) also resulted in complete loss of bacteriocin production (Fig. 4; culture J). When *cbnR*, *cbnT*, or *cbnD* was disrupted by frameshift mutations, bacteriocin production was lost (pLQ18D, pLQ18T, and pLQ18R) (Fig. 4; cultures F, G, and H, respectively), indicating that at least one or more of these genes are necessary for the production of CB2 and CBM1. This is consistent with the loss of bacteriocin production observed with pLQ11, pLQ12, and pLQ15. A slight but repeatable reduction in the amount of bacteriocin was also observed when *cbnK* (pLQ18K) was disrupted by a frameshift mutation (Fig. 4; culture I).

Immunity to CBM1 and CB2 of *C. piscicola* LV17C transformed with pCaT, pLQ18, or pLQ18 derivatives (Table 1) was determined. Introduction of frameshift mutations in *cbnR*, *cbnT*, or *cbnD* or deletions encompassing these genes or *cbnK* eliminated immunity to CBM1, in parallel with loss of bacteriocin production (Fig. 4 and 5A). However, a frameshift mutation in *cbnK* resulted in Bac^+ transformants and also eliminated the immunity to CBM1 (Fig. 4 and 5A). As expected, immunity to CB2 was lost when *cbiB2* was deleted (pLQ18E, pLQ11, and pLQ12 [Fig. 3]). However, deletions that did not affect *cbiB2* (pLQ15 and pLQ11 [Fig. 3]) and frameshift mutations in *cbnK*, *cbnR*, *cbnT*, or *cbnD* (pLQ18K, pLQ18R, pLQ18T, and pLQ18D, respectively) did not eliminate immunity to CB2 (Fig. 5B). This suggests that none of these genes is essential for CB2 immunity.

The carnobacteriocin locus is involved in the production of an additional bacteriocin-like compound(s) and an inducer factor. Sequence analysis of the cloned 10-kb fragment revealed the presence of *cbnX*, *cbnY*, and *cbnS* with predicted products that resemble precursor peptides of class II bacteriocins. To investigate whether an additional bacteriocin-like compound was encoded in pLQ18E and pLQ18B, where its

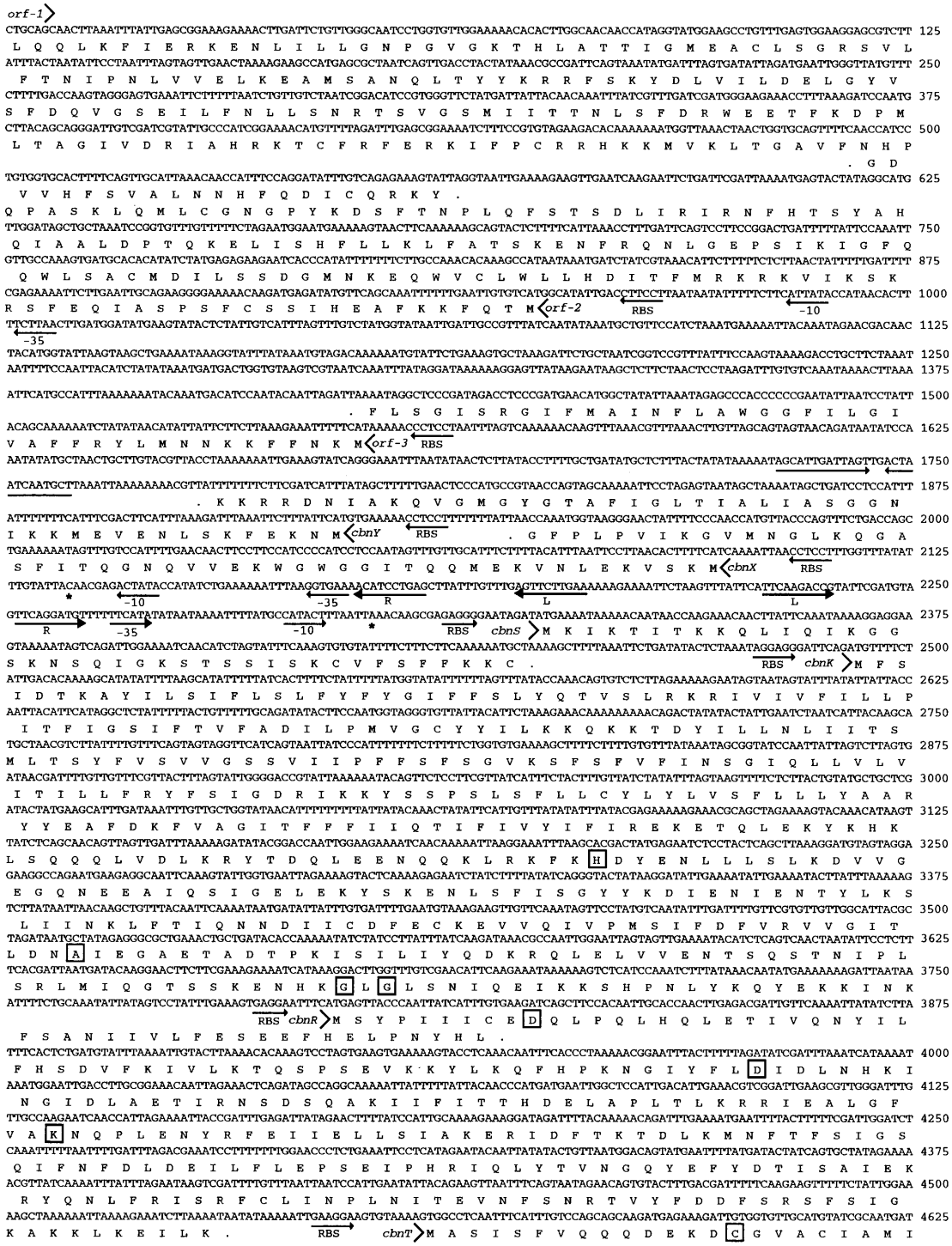

FIG. 1. Nucleotide sequence of the 10.122-kb insert from pLQ18. The deduced amino acid sequences from the identified ORFs are shown below the DNA sequence. Possible ribosome binding sites (RBS), potential −10 and −35 promoter sequences, and inverted and direct repeat (L and R, respectively) sequences are underlined. Alignment of the promoter regions is shown in Fig. 6B. Transcription initiation sites are indicated by asterisks. The DNA segment (853 bp) between nucleotides 9061 and 9913 was previously sequenced (21). The boxed amino acids (open boxes) in CbnT indicate proposed conserved key residues for the so-called Gly-Gly leader peptidase family (11, 33). The A and B conserved motifs of the ATP-binding structural domain at the C terminus of the ABC exporter proteins (9) are indicated within boxes numbered 1 and 2, respectively. The key amino acid residues of the C-terminal conserved domains I, II, and III of histidine protein kinase (28, 31) are boxed in CbnK, and the key conserved amino acid residues of the N-terminal half of response regulator proteins (28) are boxed in CbnR.

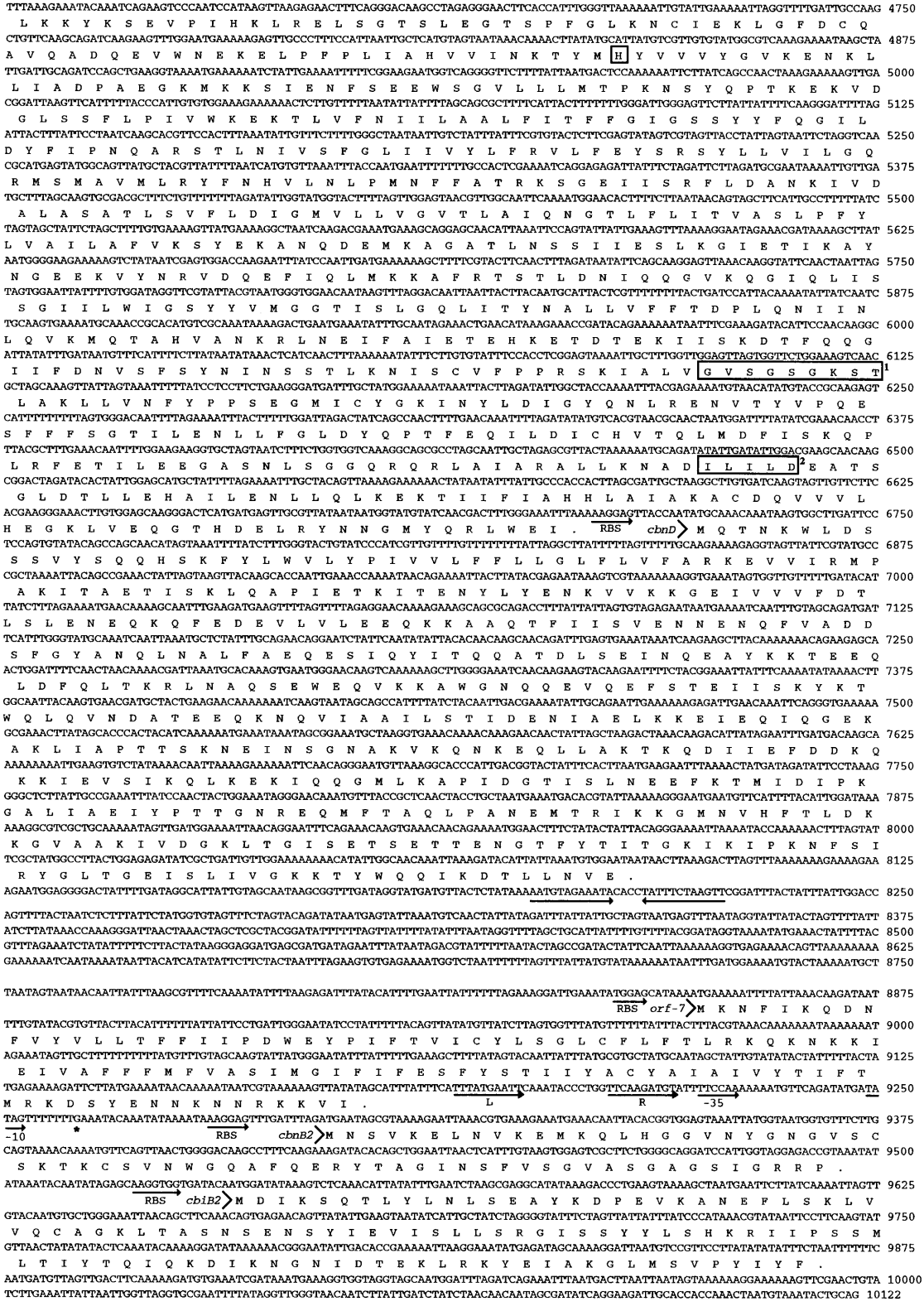

FIG. 1—*Continued.*

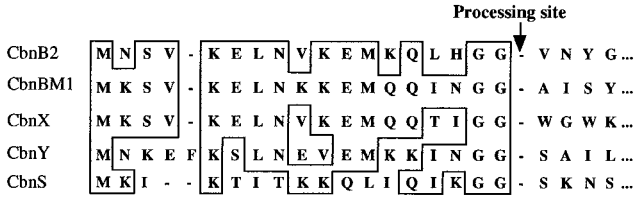

FIG. 2. Alignment of the double-glycine-type leader peptides of the bacteriocin precursors CbnB2 and CbnBM1 and the N-terminal regions of the *cbnY*, *cbnX*, and *cbnS* gene products.

activity could not be differentiated because of the production of the chromosomal bacteriocin CBM1, *C. divergens* LV13 was transformed with pLQ18, pLQ18E, and pLQ18B and the phenotype of the transformants was analyzed (Table 3). The genome of *C. divergens* LV13 does not hybridize with *cbnB2*- or *cbnBM1*-specific probes, indicating that this strain lacks the carnobacteriocin genes (data not shown). *C. divergens* LV13 harboring pLQ18 was sensitive to *C. piscicola* strains carrying plasmids with *cbnB2* deleted (Table 3, first three columns, line 3). This result was consistent with the previous observation that this host remains sensitive to CBM1 despite transformation with pLQ24 (22) and indicates that CBM1 immunity is not expressed in *C. divergens* LV13. As expected, *C. divergens* carrying pCaT or pLQ18E, with *cbiB2* deleted, was inhibited by the *C. piscicola* host carrying pLQ18, pLQ18B, or pLQ18E (Table 3, first three columns, lines 1 and 2) or *C. divergens* carrying pLQ18 (Table 3, column 4, lines 1 and 2). Furthermore, *C. piscicola* carrying pLQ18E is inhibited by *C. piscicola* carrying pLQ18 (Table 3, column 1, line 5). However, while *C. divergens* carrying the complete pLQ18 displays autoimmunity, the strain with a disruption of the immunity to CB2 (*C. divergens* carrying pLQ18E) still exhibits immunity to the undefined

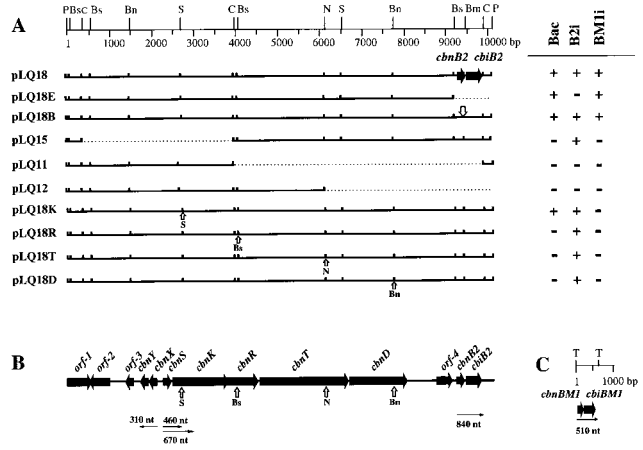

FIG. 3. (A) Schematic representation of DNA fragments cloned into pCaT. The positions of the previously sequenced *cbnB2* and *cbiB2* are indicated in pLQ18. The vertical arrow in pLQ18B indicates the site of a 31-bp deletion. Dotted lines represent deleted segments of DNA. Relevant restriction sites are indicated (P, *Pst*I; Bs, *Bst*NI; Bn, *Ban*II; S, *Sph*I; C, *Cla*I, N, *Nhe*I; Bm, *Bam*HI). The phenotypes of *C. piscicola* LV17C transformants are indicated: column 1 (Bac) indicates presence (+) or absence (−) of bacteriocin production, determined by the deferred inhibition test; columns 2 and 3 (B2i and BM1i) indicate presence (+) or absence (−) of immunity to CB2 and CBM1, respectively, determined by bacteriocin sensitivity assay. (B) Organization of the genes identified within the sequenced region. The position and size of the transcripts detected by Northern analysis (Fig. 6A) are indicated. Vertical arrows S, Bs, N, and Bn indicate the positions of frameshift mutations in *cbnK*, *cbnR*, *cbnT*, and *cbnD*, respectively. (C) Schematic diagram of the CBM1 operon. T, *Taq*I.

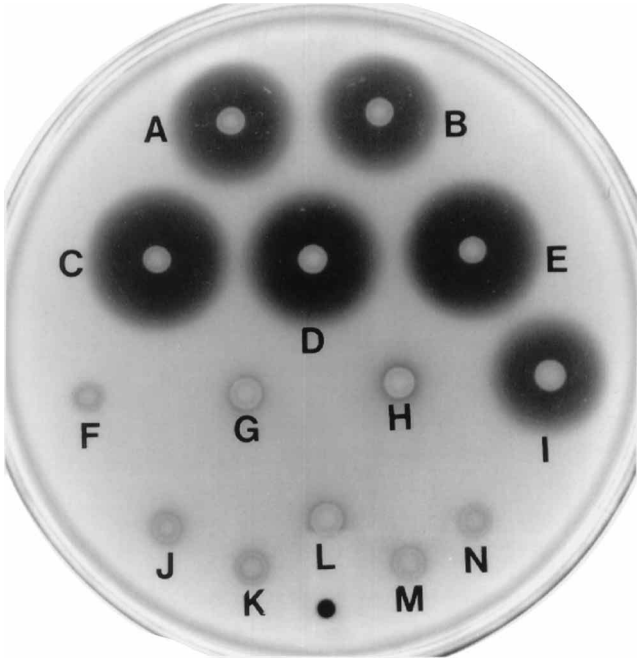

FIG. 4. Assay for bacteriocin production. Strains were inoculated onto agar plates and incubated at 25°C for 20 h before being overlaid with soft agar inoculated (1%) with the indicator strain *C. divergens* LV13 transformed with pCaT. The plates were incubated at 25°C for 16 h before the presence or absence of inhibitory activity was recorded. The letters A to L indicate the positions of *C. piscicola* LV17C transformants containing pLQ18B, pLQ18E, pLQ18′, pLQ18, pLQ24, pLQ18D, pLQ18T, pLQ18R, pLQ18K, pLQ15, pLQ12, and pLQ11, respectively. M and N indicate the positions of *C. piscicola* LV17C and *C. divergens* LV13 transformed with pCaT, respectively.

bacteriocin-like compound(s), whereas the same host carrying pCaT is susceptible (Table 3, last two columns, lines 1 and 2). This bacteriocin-like compound is still produced by *C. divergens* carrying pLQ18E or pLQ18B and is therefore not CB2. Thus, it is concluded that a bacteriocin-like compound (other than CB2 and CBM1) and an immunity protein are encoded on or regulated by the DNA fragments cloned into these pLQ plasmids.

Production of bacteriocins by *C. piscicola* LV17B is autoin-

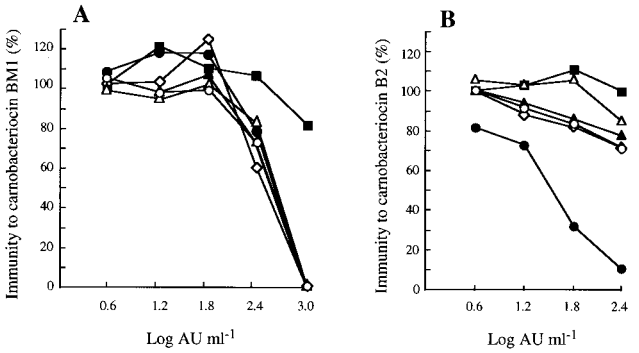

FIG. 5. Immunity to CBM1 (A) and CB2 (B) of *C. piscicola* LV17C transformed with pCaT (●), pLQ18 (■), pLQ18R (▲), pLQ18K (△), pLQ18T (◇), and pLQ18D (○). Immunity is expressed as a percentage of the growth rate in the presence of bacteriocin relative to the growth rate in the absence of bacteriocin [immunity % = (growth rate with bacteriocin/growth rate without bacteriocin) × 100]. One AU is the amount of bacteriocin required to produce a visible clearing on the lawn of the indicator strain *C. divergens* LV13. The specific activity of CB2 is 50 AU/μg, and that of CBM1 is 90 AU/μg.

TABLE 3. Bacteriocin production by transformants of *C. piscicola* LV17C and *C. divergens* LV13

Indicator strain with plasmid	Diam (mm) of zone of inhibition by producer strain with plasmid[a]					
	C. piscicola			*C. divergens*		
	pLQ18	pLQ18B	pLQ18E	pLQ18	pLQ18B	pLQ18E
C. divergens pCaT	20	18	18	19	10	10
C. divergens pLQ18E	19	19	19	19	–	–
C. divergens pLQ18	19	19	19	–	–	–
C. piscicola pLQ18B	–	–	–	ND	ND	ND
C. piscicola pLQ18E	16	–	–	ND	ND	ND

[a] Production of bacteriocin was detected by deferred inhibition test. –, no zone of inhibition; ND, not determined.

duced by CB2 (23, 27). However, transformants lacking a functional copy of *cbnB2* (pLQ18B and pLQ18E) remained Bac^+ (Table 3; Fig. 3). This observation suggested the presence of an inducer factor for carnobacteriocin production in the supernatant of these cultures, other than CB2. Culture supernatant of *C. piscicola* LV17C or *C. divergens* LV13 carrying pLQ18B or pLQ18E induced bacteriocin production in Bac^- cultures of *C. piscicola* LV17B. In contrast, culture supernatant of transformants carrying pCaT did not induce bacteriocin production. Of the frameshift mutants (pLQ18K, pLQ18R, pLQ18T, and pLQ18D), only culture supernatants of the strain carrying pLQ18K (Bac^+) induced bacteriocin production. Thus, the loss of the new unidentified inducer factor in the supernatant parallels the loss of carnobacteriocin production.

Transcription analysis of the carnobacteriocin regulon. Northern analyses of RNA isolated from induced (Bac^+) and uninduced (Bac^-) cultures of *C. piscicola* LV17B were done to study the regulation of carnobacteriocin production and immunity. All of the transcripts probed were present only in induced cultures (Bac^+), indicating that they were under common transcriptional regulation (Fig. 6A). Transcription start sites were determined by primer extension with RNA from the Bac^+ and Bac^- cultures. Primer extension products were detected only in the induced (Bac^+) culture (data not shown). Initiation sites for transcription and alignment of the promoter regions are shown in Fig. 6B. Potential −10 and −35 promoter sequences and two imperfect direct repeats were identified upstream of the transcription start sites. Based on the calculated size of the transcripts and the position of the transcription initiation sites (Fig. 6B), the following transcripts were assigned: a 510-nucleotide (nt) transcript that includes *cbnBM1* and *cbiBM1* (detected with probe 1); an 840-nt transcript that includes *cbnB2* and *cbiB2* (detected with probe 2); 670- and 460-nt transcripts, both of which include *cbnS* and part of the 5′ end of *cbnK* (detected with probe 3); and a 310-nt transcript that includes *cbnXY* (detected with probe 4). The

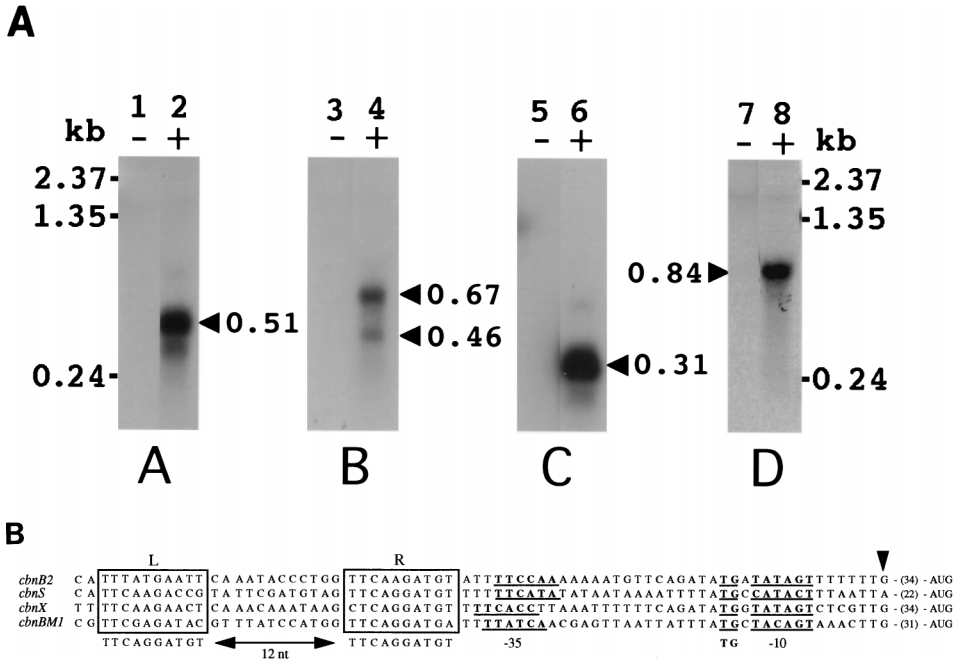

FIG. 6. (A) Northern analysis using RNA isolated from cultures induced with heat-treated culture supernatant (Bac^+) and uninduced cultures (Bac^-) of *C. piscicola* LV17B. Panels A, B, C, and D indicate hybridization to *cbnBM1-cbiBM1* (probe 1), *cbnS-cbnK* (probe 3), *cbnX-cbnY* (probe 4), and *cbnB2-cbiB2* (probe 2), respectively. The sizes of the transcripts and marker fragments are indicated in kilobases (kb). Lanes 1, 3, 5, and 7 were loaded with RNA from Bac^- cultures of *C. piscicola* LV17B; lanes 2, 4, 6, and 8 were loaded with RNA from Bac^+ cultures of *C. piscicola* LV17B. The transcripts are shown in Fig. 3B. (B) Alignment of promoter regions. Putative −10 and −35 sequences (boldface letters) are underlined. TG dinucleotides characteristic of promoters in gram-positive bacteria are indicated (20, 32). The vertical arrowhead indicates the 5′ end of the transcripts. The number of nucleotides between the 5′ end of the transcript and the initiation codon (AUG) is given in parentheses. The putative 5′ end of the *cbnBM1* transcript was determined based on sequence alignment. Direct repeats (L and R) are boxed, and the proposed consensus sequence is indicated below the boxes.

position and size of the transcripts detected are indicated in Fig. 3B. Basal level of expression of the *cbn* operons was not observed in the transcription analyses; however, it does occur. Supernatant of Bac^- cultures of *C. piscicola* LV17B incubated for more than 4 days was bacteriocin negative, but it induced bacteriocin production. Furthermore, these Bac^- cultures grew and produced bacteriocin when inoculated (2% [vol/vol]) into fresh broth (data not shown). This result indicates that low constitutive production of inducer factor(s) occurs and that it leads to accumulation of the inducer(s) and to autoinduction upon inoculation into fresh broth. This observation demonstrates the functioning of the quorum-sensing system in carnobacteriocin.

DISCUSSION

To determine the genetic requirement for bacteriocin production and immunity and the cell density-dependent mechanism involved in regulation of carnobacteriocin production, a previously cloned 10-kb DNA fragment from the plasmid pCP40 was sequenced and studied by deletion, gene inactivation, and transcription analyses. This locus contained the genetic information required for production of the plasmid-encoded CB2, the chromosomally encoded CBM1, and their immunity proteins. In addition, this locus is involved in the production of an unidentified bacteriocin-like compound and an inducer factor that plays a role in the regulation of carnobacteriocin production. Thus, *C. piscicola* LV17B not only produces several bacteriocins but contains two endogenous quorum-sensing signals that induce carnobacteriocin production.

Several new ORFs were identified in the carnobacteriocin locus, including two gene clusters, *cbnXY* and *cbnSKRTD*. Predicted products of *cbnS*, *cbnX*, and *cbnY* are class II bacteriocin-like precursor peptides. It seems likely that these genes encode the new bacteriocin(s) and (or) the inducer factor detected in this study (see below). Deduced products of *cbnK* and *cbnR* exhibit homology to proteins of bacterial Agr-type two-component signal transduction systems. Products of *cbnT* and *cbnD* show homology to ABC-transported and accessory proteins of the signal sequence-independent secretion system. More specifically, CbnT exhibits homology to ABC transporters that cleave the double-glycine leader of bacteriocin precursors (11). Frameshift mutations in *cbnR*, *cbnT*, or *cbnD* eliminated production of bacteriocins, production of inducer factor(s), and immunity to CBM1. However, immunity to CB2 was not eliminated, and it was only slightly reduced in the mutants. A frameshift mutation in *cbnK* did not eliminate bacteriocin production, inducer production, or CB2 immunity but eliminated CBM1 immunity. Because the structural and immunity genes for CBM1 are cotranscribed as an operon, it is likely that this mutant does not produce CBM1. Because of the possibility of polarity effects in genes located downstream of the mutations, it was not possible to ascertain which specific gene(s) upstream of *cbnB2* (*cbnKRTD* and *orf-4*) is required for the Bac^+ Imm^+ phenotype of *C. piscicola* LV17C carrying pLQ18. In this regard, CbnKRTD homologs are thought to be involved in production of several class II bacteriocins (4, 12, 30, 33).

It was previously shown that CB2 was the factor in the supernatant of *C. piscicola* LV17B that induced bacteriocin production (23, 27) and that when *C. piscicola* LV17B was inoculated below 10^4 CFU per ml, the resulting cultures were Bac^-, unless the growing cultures were artificially induced or grown on solid medium (27). In this study, we demonstrated that low basal production of inducer factor(s) occurs and that it leads to extracellular accumulation of the inducer to the level required for induction of bacteriocin production in Bac^- cultures or autoinduction upon subculturing. This observation indicates that a self-inducing cell density-regulated system is functional in *C. piscicola* LV17B. Furthermore, we have shown that the 10-kb fragment cloned in pLQ18 encodes (or regulates) the production of at least one additional inducer factor. The gene *plnA*, encoding the inducer plantaricin A, is located upstream of the *cbnKR* homolog *plnBCD*. Likewise, the gene *orf-1*, believed to encode the inducer of sakacin P production, is located upstream of the *cbnKR* homolog *sppKR* (12). Thus, the conserved gene arrangement found in these peptide-induced systems from lactobacilli resembles the gene arrangement *cbnSKR* in *C. piscicola* LV17B and suggests that *cbnS* encodes the precursor of an additional inducer. Transcription analysis demonstrated that the bacteriocin and immunity structural genes are cotranscribed and that the transcripts containing the carnobacteriocin structural and immunity genes are not present in uninduced (Bac^-) cultures of *C. piscicola* LV17B. Similarly, two transcripts containing *cbnS* and the 5′ end of *cbnK* and the transcript containing *cbnXY* were detected only in induced cultures. The results suggest that these transcripts are coregulated in a cell density-dependent manner. Imperfect direct repeats resembling those found in the *cbn* promoter regions are present in several promoters believed to be regulated by *agr*-like systems (6). Study of the induction factor(s) in *C. piscicola* LV17B is in progress.

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