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Characterization of the Protein Conferring Immunity to the Antimicrobial Peptide Carnobacteriocin B2 and Expression of Carnobacteriocins B2 and BM1

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Cloning of a 16-kb DNA fragment from the 61-kb plasmid of *Carnobacterium piscicola* LV17B into plasmidless *C. piscicola* LV17C restores the production of the plasmid-encoded carnobacteriocin B2 and the chromosomally-encoded carnobacteriocin BM1 and restores the immune phenotype. This fragment also has sufficient genetic information to allow the expression of carnobacteriocin B2 and its immunity in a heterologous host. The gene locus (*cbiB2*) responsible for immunity to carnobacteriocin B2 is located downstream of the structural gene for carnobacteriocin B2 and encodes a protein of 111 amino acids (CbiB2). CbiB2 was expressed in *Escherichia coli* as a fusion of the maltose-binding protein and CbiB2. The fusion protein was purified on an amylose column and cleaved with factor Xa, and pure CbiB2 was isolated by high-performance liquid chromatography. The N-terminal amino acid sequence and mass spectrometry (molecular weight [mean ± standard error], 12,662.2 ± 3.4) of the purified protein agree with the information deduced from the nucleotide sequence of *cbiB2*. Western blot (immunoblot) analysis indicates that the majority of the intracellular pool of this immunity protein is in the cytoplasm and that a smaller proportion is associated with the membrane. CbiB2 confers immunity to carnobacteriocin B2, but not to carnobacteriocin BM1, when it is expressed in homologous or heterologous hosts. No protective effect is observed for sensitive cells growing in the presence of the bacteriocin when the immunity protein is added to the medium. The purified immunity protein does not show significant binding to microtiter plates coated with carnobacteriocin B2 and is not able to inactivate the bacteriocin in solution.

In recent years, there has been growing interest in the preservation of food by lactic acid bacteria, and their commercial potential has led to the identification and characterization of several bacteriocins produced by this group of organisms. Bacteriocins are peptides or proteins with antimicrobial activity against closely related bacterial strains, but they are not active against the producer strain (23). The best characterized of these compounds is nisin A, an extensively posttranslationally modified lantibiotic which contains 34 amino acids and is produced by *Lactococcus lactis* subsp. *lactis* (21). Its nuclear magnetic resonance assignment, the genes required for its production, its mode of action, and the protein responsible for nisin immunity have been determined (13, 24, 33, 44). Although available information indicates that the bacteriocins from lactic acid bacteria exert their antimicrobial effects through action at the cell membrane (1, 9, 14, 42, 45), the mechanism of immunity that protects the producer organism against its own bacteriocin is not understood. The genetic determinants of proteins that afford protection against the nonlantibiotic bacteriocins lactococcins A, B, and M have been cloned and sequenced (40, 41). More recently, the immunity protein to lactococcin A produced by *L. lactis* subsp. *cremoris* has been purified and characterized (29, 44a).

A quite different group of bacteriocins is produced by strains of lactic acid bacteria that prevail in the microflora of chill-stored meats, packaged under vacuum or in modified atmospheres containing elevated levels of carbon dioxide (28). Carnobacteriocins B2 and BM1 (CbnB2 and CbnBM1) are small class II bacteriocins produced by *Carnobacterium piscicola* LV17B that belong to a rapidly growing family of unmodified peptides having a YGNGV sequence motif near the N terminus (31). Other members of this family include leucocin A (16), mesentericin Y105 (17), sakacins P and A (18, 38), pediocin PA-1/AcH (8, 27), and curvacin A (38). Their antimicrobial spectra include many lactic acid bacteria, as well as strains of potentially pathogenic *Enterococcus* and *Listeria* species. The genetic determinants of CbnB2 and CbnBM1 (*cbnB2* and *cbnBM1*) have been cloned, sequenced, and shown to be located on a 61-kb plasmid (pCP40) and on the chromosome, respectively (31). These carnobacteriocins are initially synthesized as precursors containing 66 and 61 amino acids that undergo posttranslational cleavage of an N-terminal extension of 18 amino acids after a Gly-Gly site to yield the mature active peptides of 48 and 43 amino acids, respectively. The phenotypic expression of both bacteriocins produced by *C. piscicola* LV17B and the immunity functions conferring protection against them are dependent on the presence of the pCP40 plasmid. Downstream of *cbnB2*, two other open reading frames (ORFs), ORF-β2 (*cbiB2*) and ORF-β3, have been sequenced from a 1.9-kb *Hin*dIII fragment of pCP40 (GenBank and EMBL data bank accession number, L29059; see reference 31 and Fig. 4). In this study, we describe the identification, characterization, heterologous expression, and purification of CbiB2, the protein conferring immunity to carnobacteriocin B2. We also report the recovery of the wild-type carnobacteriocin (Cbn^+) and immune (Imm^+) phenotypes in the cured mutant *C. piscicola* LV17C after transformation with

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant phenotype and properties	Reference or source
Bacterial strains		
C. piscicola		
LV17B	Cbn^+ Cbn^r containing pCP40[a]	3
LV17C	Cbn^- Cbn^s, plasmidless mutant derived from *C. piscicola* LV17B	3
LV17CD	LV17C containing pCaT	2
UAL26	Cbn^s	3
LQ24	LV17C containing pLQ24	This study
LQ18	LV17C containing pLQ18	This study
LQ18E	LV17C containing pLQ18E	This study
LV17Ci	LV17C containing pLQ400i	This study
UAL26i	UAL26 containing pLQ400i	This study
C. divergens		
LV13	Cbn^s	Shaw[b]
LV13pCaT	LV13 containing pCaT	2
LQ24	LV13 containing pLQ24	This study
LV13i	LV13 containing pLQ400i	This study
E. coli		
JM107	Δ(*lac proAB*) *rpsL thi endA spcB15 hsdR* Δ(*srl-recA*)306::Tn*10*(tet^r) F′[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	34
LQ400i	*E. coli* JM107 containing pLQ400i	This study
LQ300i	*E. coli* JM107 containing pLQ300i	This study
Plasmids		
pUC118	3.2-kb cloning vector, Amp^r, *lacZ′*	46
pMAL-c	6.1-kb expression vector, Amp^r, *lacZ′*	New England Biolabs Ltd.
pMG36e	3.4-kb expression vector, Em^r	43
pCaT	8.5-kb cloning vector, Cm^r	20
pCP40	61-kb plasmid conferring Cbn^+ Imm^+ phenotype	3
pLQ24	pCaT containing a 16-kb fragment from pCP40	This study
pLQ18	pCaT containing a 9.5-kb fragment from pCP40	This study
pLQ18E	pLQ18 with *cbnB2* and *cbiB2* deleted	This study
pLQ300i	pMAL-c containing *malE-cbiB2* fusion	This study
pLQ400i	pMG36e containing 442-bp insert with *cbiB2*	This study
pLQ5.21	pUC118 containing a 1.9-kb *Hin*dIII fragment of pCP40	31

[a] Cbn, carnobacteriocin(s).
[b] Supplied by B. G. Shaw, Institute of Food Research, Langford, Bristol, United Kingdom.

DNA fragments derived from pCP40, and we describe the expression of carnobacteriocin B2 in a heterologous host.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Carnobacterium* strains were grown in APT broth (Difco) as previously described (3). *Escherichia coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar (34). Medium containing ampicillin (100 μg/ml), erythromycin (100 μg/ml for *E. coli* and 5 μg/ml for *Carnobacterium* strains), or chloramphenicol (5 μg/ml) was used for growth and selection of transformants. Bacteriocin production was determined by the spot-on-lawn test (3). Different strains of lactic acid bacteria were challenged with the culture supernatant of the strains to be tested for bacteriocin production.

Bacteriocin sensitivity assay. The sensitivity of selected strains to carnobacteriocins B2 and BM1 was determined by the spot-on-lawn test (spotting 0.2 μg of bacteriocin) or in microtiter plates. In the latter case, the wells were loaded with serial fourfold dilutions of the carnobacteriocin B2 (0.25, 1, 4, and 16 μg/ml) in 150 μl of APT broth inoculated with a fresh culture (1% inoculum). The plates were incubated at 25°C, and the A_{590} was measured at 30-min intervals. The effect of bacteriocin on cell growth in the presence of purified immunity protein (100 μg/ml) or bovine serum albumin (BSA; 100 μg/ml) was evaluated. The bacteriocins used in the tests were purified by methods previously described (31).

DNA isolation, manipulation, and sequencing. Isolation of plasmid DNA from *Carnobacterium* strains and *E. coli* was done according to methods previously described (3, 34). Restriction digestion, exonuclease III treatment, 5′ labeling with [γ-^{32}P]ATP, Southern hybridization analyses on nylon membranes, and DNA ligation were done by standard procedures (34). Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and exonuclease III were purchased from Boehringer Mannheim (Dorval, Quebec, Canada). Transformation of *E. coli* competent cells and strains of lactic acid bacteria and selection and screening of transformants were done by established methods (4, 34). DNA was sequenced with *Taq* DNA polymerase and fluorescent dideoxy-chain terminators and analyzed on an Applied Biosystems 373A DNA sequencer. The recombinant plasmid pLQ300i was used as a template (Table 1), and the reactions were primed with forward and reverse primers for pMAL vectors (New England Biolabs, Ltd., Mississauga, Ontario, Canada).

Subcloning of *cbiB2* and construction of the *malE-cbiB2* fusion. A 442-bp *Bam*HI-*Cla*I fragment from pLQ5.21 containing the last 21 bp of *cbnB2*, the intergenic region between *cbnB2* and *cbiB2*, *cbiB2*, and 48 bp downstream of *cbiB2* was subcloned into pUC118, excised with *Sac*I-*Hin*dIII, and subcloned into pMG36e to create pLQ400i (Table 1; Fig. 1). pLQ400i was introduced into selected strains of lactic acid bacteria, and the sensitivity of the transformants to the carnobacteriocins was studied. For the construction of the *malE-cbiB2* fusion, *cbiB2* was amplified by PCR with two specific primers synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer. ImmF (5′ XATGGAT ATAAAGTCTCAAAC 3′, where X is a phosphate), based on the 5′ nucleotide sequence of *cbiB2*, and ImmR (5′ CGCTCTAGATTAGAAATATATATAAG GAAC 3′), based on the 3′ nucleotide sequence of *cbiB2* and containing a 5′ overhang of 9 nucleotides with an *Xba*I restriction site, were used to prime the forward and reverse reactions, respectively. The amplified fragment was digested with *Xba*I and cloned into pMAL-c linearized with *Stu*I and *Xba*I to create a fusion in the correct translational reading frame between the *E. coli* maltose-binding protein gene (*malE*) and *cbiB2* in pLQ300i (Table 1).

Immunity protein purification. Carnobacteriocin B2 immunity protein (CbiB2) was purified from 1 liter of *E. coli* LQ300i culture grown in LB broth with 0.2% glucose and 100 μg of ampicillin per ml at 37°C to an optical density at 600 nm of 0.5. The expression, affinity purification, and factor Xa cleavage of the fusion protein were done as recommended in the instruction manual (28a). No denaturation of the fusion protein was done before factor Xa cleavage. CbiB2 was purified by reversed-phase high-performance liquid chromatography (HPLC) with a C_8-VYDEC column (10 by 250 mm, 10-μm particle size, 300-Å [30.0-nm] pore size) and a gradient from 38.5 to 45.5% of acetonitrile in 0.1% trifluoroacetic acid (flow rate, 2.5 ml/min [monitored at 218 nm]). Purified samples of

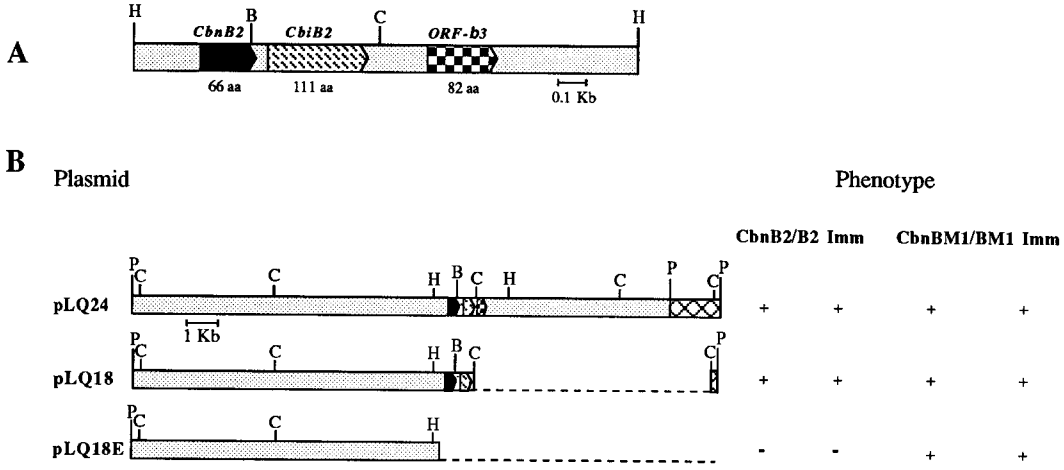

FIG. 1. Schematic representations and partial restriction maps of the previously sequenced 1.9-kb *Hin*dIII fragment from pCP40 containing *cbnB2* and *cbiB2* (A) and of the inserts present in pLQ24, pLQ18, and pLQ18E (B). The phenotypes of the *C. piscicola* LV17C hosts are indicated. Restriction sites: P, *Pst*I; C, *Cla*I; B, *Bam*HI, and H, *Hin*dIII. aa, amino acids. The dashed lines indicate deleted segments.

CbiB2 were lyophilized and stored at −20°C. Protein concentration was estimated by a modification of the method of Lowry (26).

N-terminal sequence analysis and mass spectrometry. The N-terminal amino acid sequencing of CbiB2 was done by Edman degradation on an automated gas phase sequencer (Applied Biosystems model 470A) with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). The mass spectrometry data for the purified immunity protein were obtained from the positive ion electrospray analysis done at the National Research Council (Canada) Plant Biotechnology Institute (Saskatoon, Saskatchewan, Canada) on a Fisons Trio 2000 mass spectrometer equipped for electrospray. The lyophilized sample was dissolved in 50% acetonitrile–50% of a 1% formic acid solution and loop injected (10 μl) at a flow rate of 6 μl/min.

Antiserum against carnobacteriocin B2 immunity protein. Two rabbits were injected at 3-week intervals, three times with 200 μg of purified fusion protein and once with 100 μg of purified CbiB2 (final injection). Immunogens were prepared in Ribi Adjuvant for rabbits as recommended by the suppliers (Immuno Chem Research Inc., Hamilton, Mont.). The immunization procedure was done in accordance with the animal welfare guidelines of the University of Alberta. The antibodies against CbiB2 in the serum collected after the last injection were purified by affinity binding to an antigen column. To obtain the CbiB2 column, the immunity protein was electroeluted after electrophoresis and covalently linked to Sepharose CL-6B (Pharmacia) by established methods (19, 37).

Preparation of cytoplasmic, membrane, and extracellular protein fractions. Cytoplasmic (soluble) and membrane fractions were prepared on the basis of previously reported methods (22). Cells from 1-liter cultures of *C. piscicola* LV17B and LV17C were grown to early stationary phase and harvested by centrifugation (13,000 × *g* for 10 min). The supernatants were stored at −20°C (extracellular protein fraction). The cells were washed with 150 ml of 10 mM Tris-HCl buffer, pH 8 (solution A), harvested by centrifugation, resuspended in 60 ml of the same buffer containing 20% sucrose, 10 mM $MgSO_4$, 4 mg of lysozyme (Sigma, Chemical Company, St. Louis, Mo.) per ml, and 15 μg of mutanolysin (Sigma) per ml (solution B), and incubated for 1 h at 37°C. After the incubation, the presence of protoplasts (ca. 90%) was confirmed by phase-contrast microscopy and lysis was achieved by a combination of osmotic shock and treatment in a French pressure cell. Protoplasts harvested by centrifugation (20,000 × *g* for 15 min) were resuspended in 300 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgSO_4$, 5 μg of deoxyribonuclease I (Sigma) per ml, and 5 μg of ribonuclease A (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada) per ml (solution C), and the suspension was passed through a French pressure cell at 1,300 lb/in^2. The lysates were centrifuged twice at 8,000 × *g* for 15 min, and the resulting supernatants were centrifuged at 100,000 × *g* for 90 min. The cleared supernatants were stored at −20°C (cytoplasmic fraction). The pellets were resuspended in 20 ml of solution C without nucleases, and the suspensions were centrifuged (100,000 × *g* for 90 min). The pellets (membrane fraction) were resuspended in 6 ml of solution C without nucleases and stored at −20°C.

Glucose-6-phosphate dehydrogenase (25) and NADH oxidase (10) activities in the fractions were determined spectrophotometrically by previously described methods (6). The reaction volume was 300 μl, and the reaction mixture contained up to 250 μl of sample. Some reactions were performed in the presence of 1% Triton X-100 to solubilize the membrane fraction. The assays were done in triplicate, and the standard errors were less than 10% of the triplicate mean.

Washing of protoplasts and membranes with high-ionic-strength solutions. Protoplasts from 100 ml of culture prepared as described above in solution B were harvested (20,000 × *g* for 15 min) and resuspended in 4 ml of 10 mM Tris-HCl buffer, pH 8, containing 20% sucrose, 10 mM $MgSO_4$, and 1 M NaCl. After being shaken at 50 rpm for 20 min, the suspension was centrifuged, and the clear supernatant (protoplast wash) was stored at −20°C. Cell membranes suspended in solution C prepared from 150 ml of culture, as described above, were harvested (100,000 × *g* for 90 min) and resuspended in 4 ml of solution C or in the same solution containing 1 M NaCl. The membranes were harvested and washed in the same way four more times. The washed membranes were stored at −20°C.

Bacteriocin immunity binding analysis. The binding of the immunity protein (30 μg/ml of solution) to microtiter plate wells coated with carnobacteriocin B2 after 1 h of incubation at room temperature was determined by standard indirect enzyme-linked immunosorbent assay (ELISA) with antibodies against CbiB2 as the primary antibody and goat antirabbit peroxidase conjugated antibodies as the detection system. The assays were done by standard procedures (39). Similarly, the binding of immunity protein biotinylated via biotin-*N*-hydroxysuccinimide in molar ratio 1:3 to polystyrene wells coated with bacteriocin was determined with peroxidase-streptavidin conjugates. Labeling with biotin and detection with peroxidase-streptavidin were done according the supplier's recommendations (ICN Biomedicals).

To determine if the immunity protein was able to inactivate the bacteriocin in solution, 7.5 μg of carnobacteriocin B2 was mixed with 10 or 100 μg of immunity protein in 100 μl of phosphate buffer, pH 7. Controls with BSA instead of immunity protein and with bacteriocin alone were also prepared. The inhibitory activity of the samples was determined by the spot-on-lawn test before and after incubation at 22°C for 24 h.

Polyacrylamide gel electrophoresis and Western blot analysis. Sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS–12% PAGE) was performed by standard procedures (34). Western blot (immunoblot) analyses were done with the ECL Western Blotting Detection System as recommended by the suppliers (Amersham Canada Ltd., Oakville, Ontario, Canada). The proteins were electroblotted onto nitrocellulose membranes (0.2-μm pore size) with carbonate blotting buffer containing 10 mM $NaHCO_3$ and 3 mM Na_2CO_3, pH 9.9, in 20% methanol (12). The proportion of the immunity protein represented by the signals on the Western blots was estimated with a gel scanner (2222-020 Ultroscan XL; Pharmacia LKB Biotechnology).

RESULTS

Expression of carnobacteriocins B and identification of carnobacteriocin B2 immunity protein. The plasmids pLQ24, carrying a *Pst*I fragment of approximately 16 kb derived from pCP40, and pLQ18, carrying a smaller fragment of approximately 9.5 kb derived from pLQ24 by partial digestion with *Cla*I, restored the production of carnobacteriocins B2 and BM1 and the immune phenotypes in *C. piscicola* LV17C when the plasmids were electrotransformed into the strain. Similarly, introduction of pLQ24 into the heterologous host *Carnobac-*

TABLE 2. Production of carnobacteriocins B by strains of *C. piscicola*

Indicator strain	Presence of zone of inhibition with indicated producer strain[a]				
	C. piscicola				*C. divergens* LQ24
	LV17B	LQ24	LQ18	LQ18E	
C. piscicola					
LV17CD	+	+	+	+	ND[b]
LQ24	−	−	−	−	ND
LQ18	−	−	−	−	ND
LQ18E	+	+	+	−	ND
C. divergens LV13pCaT	+	+	+	+	+

[a] Antimicrobial activity was determined by spotting 10 μl of heat-treated supernatant (65°C, 15 min) of the producer strain culture onto an overlay inoculated (1%) with the specific indicator strain. The presence (+) or absence (−) of zones of inhibition was assessed after 18 h of incubation.
[b] ND, not determined.

terium divergens LV13 provided the genetic information necessary to give transformants that produced carnobacteriocin B2 that showed immunity to carnobacteriocin B2 but not to carnobacteriocin BM1. *C. piscicola* LQ24 and LQ18 displayed a fourfold increase in inhibitory activity of the supernatant compared with that of the wild type, *C. piscicola* LV17B. Although there is no precise information about the copy numbers of pCP40 and pCaT, pLQ24 and pLQ18 appear to have higher copy numbers than pCP40, judging by the intensity of the bands in agarose gel electrophoresis. This might account for the increase in inhibitory activity by these transformants. *C. piscicola* LV17C carrying pLQ18E, a derivative of pLQ18 with an exonuclease III deletion encompassing *cbnB2* and *cbiB2*, did not produce CbnB2 and was sensitive to it, but it remained immune to and produced CbnBM1 (Tables 2 and 3 and Fig. 1). The 9.5-kb insert of pLQ18 contains the genetic information necessary to restore the expression of CbnB2 and its immunity protein and restore the expression and immunity function of the chromosomally encoded CbnBM1. This suggests that complementing *trans*-acting factors encoded in the plasmid pCP40 that are required for expression of the chromosomal bacteriocin are encoded upstream of *cbnB2* in the 9.5-kb insert of pLQ18. These results also indicate that CbiB2, a protein of 111 amino acids, could provide immunity to CbnB2. To verify the immunity function of CbiB2, pLQ400i, containing the structural gene of the suspected immunity protein under the control of a constitutive promoter of the shuttle vector pMG36e, was transformed into the homologous host *C. piscicola* LV17C and into the heterologous hosts *C. piscicola* UAL26 and *C. divergens* LV13, all of which are sensitive to carnobacteriocins B2 and BM1. Spot-on-lawn tests showed that the transformants are immune to carnobacteriocin B2; however, they are not immune to carnobacteriocin BM1 (Table 3).

TABLE 3. Sensitivity of selected strains to carnobacteriocins B2 and BM1

Indicator strain	Sensitivity to carnobacteriocin[a]:	
	B2	BM1
C. piscicola		
LV17B	−	−
LV17C	+	+
UAL26	+	+
LQ24	−	−
LQ18	−	−
LQ18E	+	−
LV17Ci	−	+
UAL26i	−	+
C. divergens		
LV13	+	+
LQ24	−	+
LV13i	−	+

[a] Sensitivity to carnobacteriocins B2 and BM1 was determined by spotting 10 μl of bacteriocin solution (20 μg/ml in peptone water) onto an overlay inoculated (1%) with the specific indicator strain. The presence (+) or absence (−) of zones of inhibition was assessed after 18 h of incubation.

The growth of the transformants (LV17Ci, LV13i, and UAL26i), the parental strains (LV17C, LV13, and UAL26), and the carnobacteriocin producer strain LV17B (Table 1) was evaluated in the presence of several concentrations of carnobacteriocin B2. No difference in growth of the producer strain was observed with the range of bacteriocin concentrations tested (0, 0.25, 1, 4, and 16 μg/ml). However, differences in the levels of sensitivity to the bacteriocin between the parental strains and the strains transformed with pLQ400i were observed. *C. divergens* LV13i and *C. piscicola* LV17Ci and UAL26i display increased immunity compared with the parental strains (Fig. 2). Although growth of the sensitive strains was reduced or suppressed in the presence of the bacteriocin during the first 20 h of incubation, a final reading of the optical density after 48 h indicated that all of the strains had grown to maximum population (data not shown). To verify the presence of carnobacteriocin B2, the bacteriocin activity was monitored after 30 and 48 h of incubation. The bacteriocin concentrations at 30 and 48 h were the same and represented approximately half of the initial concentration. To determine if the presence of purified immunity protein in the medium protected sensitive strains against the bacteriocin, the immunity protein (100 μg/ml) was added to the medium containing carnobacteriocin B2 (8 μg/ml) and growth of the inoculum was monitored. The same experiment was performed with BSA (100 μg/ml) instead of immunity protein, as a control. The addition of neither the immunity protein nor the BSA protected the sensitive strains against the bacteriocin in the broth. In all of these cases, the same extended lag phase was observed (data not shown). Preincubation for 24 h of bacteriocin alone, with immunity protein, or with BSA did not have any effect on the inhibitory activity evaluated by the spot-on-lawn test. The inhibitory activity of all of the samples was the same (4,000 arbitrary units/ml).

Purification of carnobacteriocin B2 immunity protein. CbiB2 was expressed in *E. coli* as a stable hybrid protein composed of the maltose-binding protein encoded on pMAL-c and 111 amino acids of CbiB2. The proper DNA fusion and the nucleotide sequence of the fused *cbiB2* of pLQ300i were confirmed by DNA sequencing. The fusion protein was expressed in, and purified from, the cytoplasm of *E. coli* LQ300i, and it represented approximately 7 to 9% of the total cellular protein. After cleavage with factor Xa, which did not require previous denaturation of the fusion protein, the immunity protein was isolated and purified by reversed-phase HPLC. The retention times of the maltose-binding protein, the undigested fusion protein, and the immunity protein were 18.9, 21.4, and 23.0 min, respectively. The final yield of CbiB2 was 4 to 5 mg/liter of culture. To verify the proper cleavage with factor Xa at the N terminus of CbiB2, the first 5 amino acids of the protein sequence were determined by Edman degradation. The amino acid sequence Met-Asp-Ile-Lys-Ser-, which coincides with the amino acid sequence predicted from *cbiB2*, was

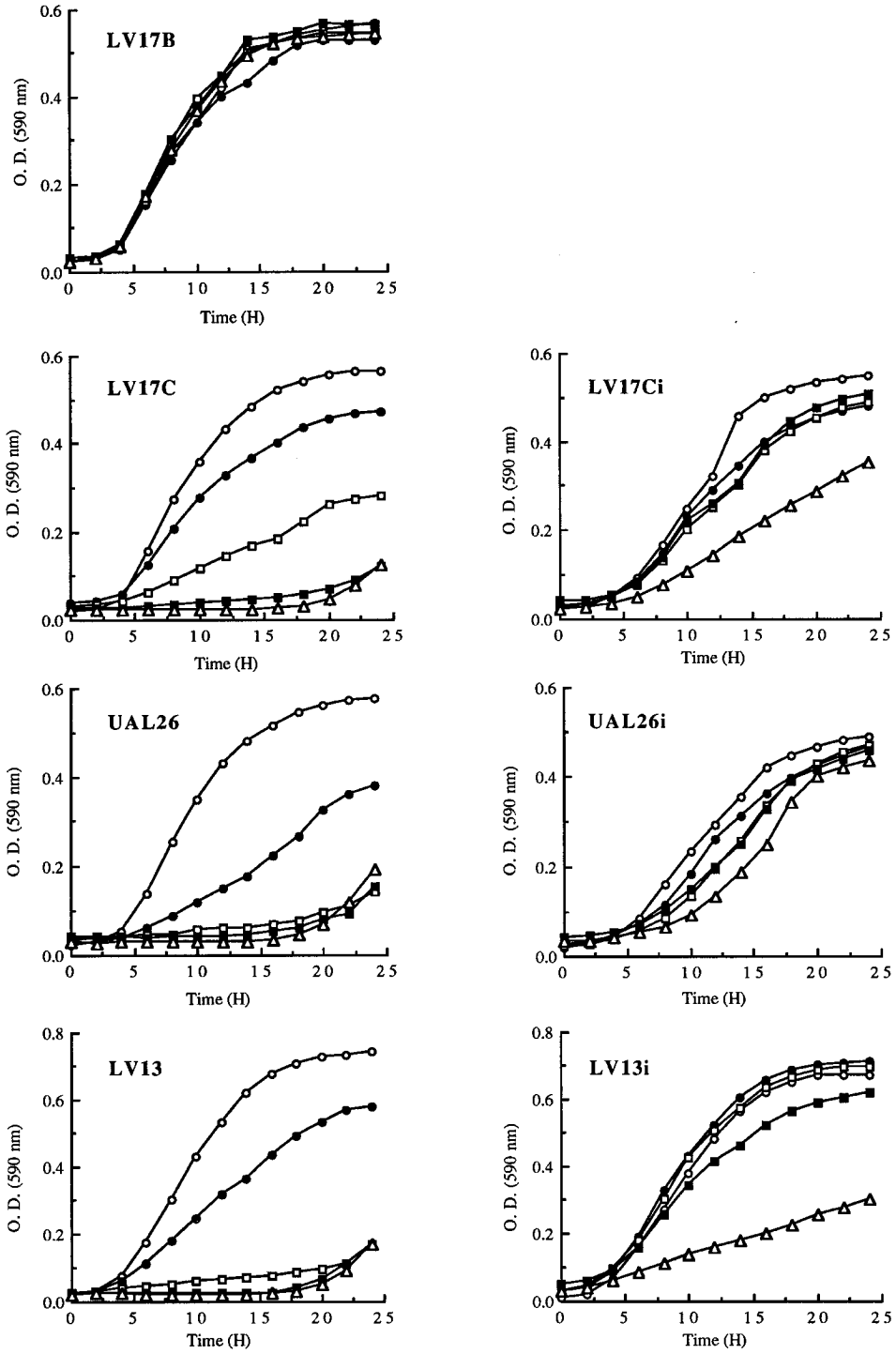

FIG. 2. Kinetics of growth of selected *Carnobacterium* strains in the presence of carnobacteriocin B2. The plots show the changes in optical density (O.D.) of selected cultures in the absence (○) or presence (●, 0.25; □, 1; ■, 4; and △, 16 μg/ml) of the bacteriocin.

found. To ascertain that CbiB2 was fully translated and to ascertain whether it was posttranslationally modified in *E. coli*, the molecular mass of the purified protein was determined by mass spectrometry. The molecular weight of 12,662.2 ± 3.4 (Fig. 3) is in agreement with the molecular weight of 12,666.50 predicted from the amino acid sequence deduced from *cbiB2* (31). Experiments to detect the binding of the immunity protein or biotinylated immunity protein to microtiter plates coated with carnobacteriocin B2 were done. No binding was detected in either of two independent experiments under the conditions of the assays (data not shown).

Cellular localization of CbiB2. To establish the cellular localization of CbiB2, the proteins from the cytoplasm and cell membrane of *C. piscicola* LV17B were analyzed by Western

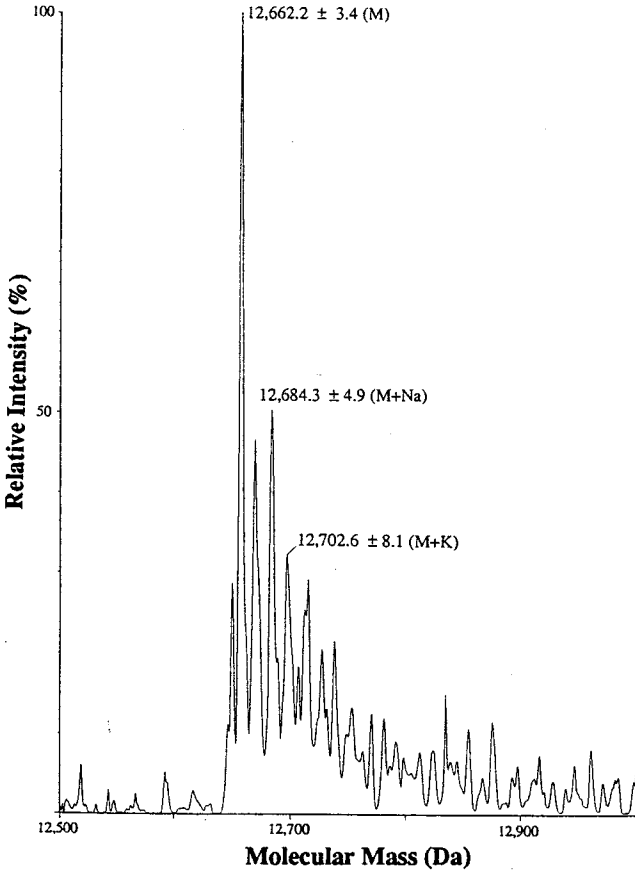

FIG. 3. Molecular mass of carnobacteriocin B2 immunity protein calculated from the positive ion electrospray mass spectra. Values shown are means ± standard errors.

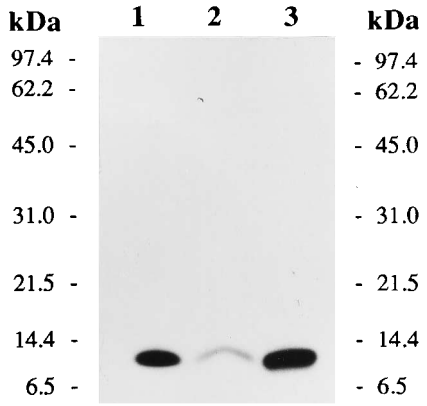

FIG. 4. Western blot analysis of membranes and cytoplasmic proteins from *C. piscicola* LV17B. Lane 1 was loaded with approximately 0.1 μg of purified CbiB2, and lanes 2 and 3 were loaded with 90 μg of proteins from *C. piscicola* LV17B membrane and cytoplasmic fractions, respectively.

blot with purified antibodies against CbiB2. The total proteins in the cytoplasmic and membrane fractions produced with the cell fractionation protocol were approximately 130 and 13 mg, respectively. Cytoplasmic and membrane proteins of *C. piscicola* LV17C were also analyzed as negative controls to determine the specificity of the antibodies. No signal was detected (data not shown). The results revealed the presence of a protein in the cytoplasmic and membrane fractions of *C. piscicola* LV17B with the same electrophoretic mobility as that of the purified immunity protein. The protein had an apparent molecular mass of 9.5 kDa, on the basis of SDS-PAGE. The majority of CbiB2 is present in the cytoplasm, and a small proportion is present in the membrane fraction of *C. piscicola* LV17B. Scanning of the X-ray film indicated that the proportions of the immunity protein in the cytoplasmic and membrane fractions were 92 and 8%, respectively (Fig. 4).

To monitor the possible contamination of the membranes with components from the cytoplasmic fraction, the glucose-6-phosphate dehydrogenase activity of the fractions was evaluated. The specific activities of the cell lysate before ultracentrifugation and of the cytoplasmic fraction were 10.4 and 10.2 mU/mg, respectively. The presence of Triton X-100 in the reaction mixture did not affect the enzymatic activities of these fractions. No activity was detected in the membrane fraction, regardless of the absence or presence of Triton X-100. To confirm the enzymatic activity of the membranes, NADH oxidase activity was also determined. The specific activities of the membrane and cytoplasmic fractions were 71.8 and 26.3 mU/mg, respectively.

The presence of the immunity protein was also evaluated in the culture broth, the cell wash (solution A), the sucrose solution (solution B), the protoplast wash, and membranes washed with solution C with or without NaCl. Traces of the immunity protein were detected in the first four fractions only by exposure of the X-ray film for prolonged periods of time (data not shown). The presence of immunity protein in these fractions is probably due to some cell lysis, as indicated by the glucose-6-phosphate dehydrogenase activities in some of these fractions: 0.04 and 1.4 mU/mg for the sucrose solution and protoplast wash, respectively. The membranes washed with high-ionic-strength solution (1 M NaCl) contained approximately the same amount of immunity protein as those washed with solution C without NaCl. Scanning indicated that the proportions of the immunity protein in the membranes washed with or without NaCl were 62 and 38%, respectively.

DISCUSSION

C. piscicola LV17B produces two class II bacteriocins, carnobacteriocin B2, encoded on a 61-kb plasmid, and carnobacteriocin BM1, encoded on the chromosome (23, 31). A 16-kb fragment from the plasmid has not only the genetic information required to restore the wild-type Cbn^+ Imm^+ phenotype in the homologous host but also that required to confer a $CbnB2^+$ $CbnB2Imm^+$ phenotype on a heterologous host. This fragment encodes proteins that appear to form part of a secretion, transcriptional activation, or processing system required for the Cbn^+ Imm^+ phenotype. Specific proteins that belong to the HlyB family of ATP-dependent translocators (7) that may be involved in the transport of the class II bacteriocins lactococcin A and pediocin PA-1/AcH have been identified (8, 27, 36), and proteins with homology to the elements of the two-component signal transduction system (35) required for the production of the class II bacteriocins sakacin A and plantaricin A have also been reported (5, 11). Preliminary results have revealed the presence of several ORFs upstream of *cbnB2*, and one of them encodes a protein of 965 amino acids with homology to ATP-dependent transmembrane translocators. The possible function of this ORF in bacteriocin secretion is under investigation.

Deletion and subcloning experiments allowed the identification of *cbiB2* as the gene encoding the protein that confers

immunity against carnobacteriocin B2. CbiB2 is a protein consisting of 111 amino acids, with a calculated pI of 9.31 and no homology to other proteins found in data banks (31). Proteins that confer immunity to lactococcins A, B, and M are also relatively small, containing 98, 91, and 154 amino acids, respectively, and they do not have homology with other reported proteins (40, 41). CbiB2 is able to protect homologous and heterologous hosts against the activity of carnobacteriocin B2. However, the level of immunity of the transformed strains expressing CbiB2 is not as great as that of the wild-type strain. This could be due to poor levels of transcription of *cbiB2* under the control of the pMG36e promoter, or it might indicate that another gene product, in addition to CbiB2, is required for full immunity. The nature of the resistance to carnobacteriocin B2 manifested by the sensitive strains after 20 h of incubation in the presence of bacteriocin is unknown. The resistance is shown by homologous and heterologous strains, indicating that this mechanism of resistance is not exclusive to the carnobacteriocin producer strain and that it is unrelated to the immunity conferred by CbiB2. The occurrence of resistant strains within a sensitive bacterial population is a common phenomenon for bacteriocins produced by lactic acid bacteria (15, 30, 32).

Although carnobacteriocins B2 and BM1 have significant amino acid homology (34% identity) and similar spectra of antimicrobial activity (31), CbiB2 does not confer immunity to carnobacteriocin BM1. Interestingly, *cbiBM1*, located downstream of *cbnBM1*, encodes a basic protein (pI 9.51) of 88 amino acids with 19% identity with CbiB2 (31). The possible immunity function of CbiBM1 against CbnBM1 is under investigation. The gene *cbiB2* is located downstream of *cbnB2*. This genetic arrangement is the same as that found for the genes responsible for immunity to lactococcins A, B, and M, which are also located downstream of the bacteriocin structural gene and form part of the bacteriocin operons (40, 41). Likewise, downstream of the genes for the class II bacteriocins leucocin A and pediocin PA-1/AcH there are ORFs encoding basic proteins of 113 and 112 amino acids, respectively, with limited amino acid homology to CbiB2 (12% identity) that have been reported as possible immunity proteins (8, 16, 27). However, the function of these proteins has not been established.

The lack of a specific assay or known biological activity in vitro that would allow the direct detection of immunity proteins makes their purification particularly difficult. In the case of the carnobacteriocin B2 immunity protein, its expression in *E. coli* as a fusion protein proved to be an effective method of purification. Furthermore, the expression in *E. coli* should allow facile isotopic labeling of CbiB2 for study of the solution structure of the protein by nuclear magnetic resonance.

The majority of the intracellular pool of CbiB2 is located in the cytoplasm, and a small proportion is associated with the membrane. Considering the result of the Western blot analysis and the protein contents of the cytoplasmic and membrane fractions, the immunity protein present in the membranes represents no more than 1% of the cellular pool. This result contrasts with the results obtained for the lactococcin A immunity protein, which was found in approximately the same proportions in the cytoplasmic and membrane fractions of immune strains (29, 44a). Although the immunity protein could have some affinity for the membrane surface because of the protein's positive charge at physiological pH, the nature of the interaction of CbiB2 with the membrane is unclear. The membranes had similar amounts of immunity protein after several washes with high- or low-ionic-strength solutions. This result could be explained by an assumption that the protein is trapped within the membrane vesicles. During protoplast lysis, the protein might be selectively retained inside the vesicles because of some affinity for membrane components instead of being removed with the rest of the cytoplasmic components. This result could also be explained if a small fraction of immunity molecules is embedded in the membrane. Although several hydrophobic segments of CbiB2 can be identified, no apparent transmembrane-spanning domains (31) can be predicted from the amino acid sequence. More recently, lactococcin A immunity protein, which has no transmembrane-spanning segment predicted from its sequence, has been shown to have its C terminus located on the outside of the cell and a possible transmembrane α-amphiphilic helix from residues 27 to 49 that could be responsible for anchoring the protein to the membrane (44a). Carnobacteriocin B2 immunity protein is not an extracellular protein, no signal sequence can be predicted from its amino acid sequence, and it cannot be removed from protoplasts with high-ionic-strength solutions.

Carnobacteriocin B2, like other bacteriocins, targets the cytoplasmic membrane of sensitive cells, and it causes dissipation of the proton motive force with leakage of intracellular components through the probable formation of pores (39a). Expression of CbiB2 within the bacterial cell protects against the antimicrobial action of CbnB2 applied externally, but the addition of purified CbiB2 to the medium does not protect the sensitive strains. This is in agreement with the cellular localization of CbiB2. These results, together with the apparent low binding affinity of CbiB2 with the bacteriocin and the inability of CbiB2 to inactivate the bacteriocin in solution, suggest that the immunity protein and the bacteriocin do not interact directly in aqueous solution. However, the possibility that the purified immunity protein is not in its native conformation cannot be eliminated. It seems unlikely that the immunity protein directly inhibits the interaction of the extracellular bacteriocin with the membrane. Rather, CbiB2 may interfere with the formation of a functional pore complex in the membrane or it may block the functional pore to prevent the efflux of intracellular components by interacting with the portion of the pore structure facing the cytoplasm. A key feature of the mode of action of the immunity protein might be its interaction with a receptor protein that binds both the bacteriocin and its immunity protein, as might be the case for the lactococcin A immunity protein (44a). Current studies on the interaction of CbnB2 and CbiB2 in membrane environments and the manipulation of their genes to identify key residues involved in biological activity will facilitate understanding of the mechanism of antimicrobial action as well as immunity.

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