Effect of Amino Acid Substitutions on the Activity of Carnobacteriocin B2

OVERPRODUCTION OF THE ANTIMICROBIAL PEPTIDE, ITS ENGINEERED VARIANTS, AND ITS PRECURSOR IN ESCHERICHIA COLI*

(Received for publication, August 14, 1996, and in revised form, October 24, 1996)

**Luis E. N. Quadri**, **Liang Z. Yan**, **Michael E. Stiles**, and **John C. Vederas**

From the **Department of Chemistry and the Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta T6G 2G2, Canada**

Carnobacteriocin B2, a 48-amino acid antimicrobial peptide containing a YNGV motif that is produced by the lactic acid bacterium *Carnobacterium piscicola* LV17B, was overexpressed as fusion with maltose-binding protein in *Escherichia coli*. This fusion protein was cleaved with Factor Xa to allow isolation of the mature bacteriocin that was identical in all respects to that obtained from *C. piscicola*. Similar methodology permitted production of the precursor precarnobacteriocin B2 (CbnB2P), which has an 18-amino acid leader, as well as six mutants of the mature peptide: CbnF3 (Tyr3 → Phe), CbnS33 (Phe33 → Ser), CbnI34 (Val34 → Ile), CbnB2P (Val37 → Ile), CbnG46 (Arg46 → Gly), and Cbn28 (truncated frameshift mutation: (carnobacteriocin B2 1–28) + ELTHL). Examination of these compounds for antimicrobial activity showed that although CbnI34, CbnI37, and CbnG46 were fully active, CbnB2P, CbnF3, CbnS33, Cbn28, and all of the fusion proteins had greatly reduced or no antimicrobial activity. Expression of the immunity protein that protects against the action of the parent carnobacteriocin B2 in a previously sensitive organism also protects against the active mutants. Because carnobacteriocin B2 also acts as an inducer of bacteriocin production in *C. piscicola*, the ability of the precursor CbnB2P and the mutants to exert this effect was examined. All were able to induce Bac– cultures and reestablish the Bac+ phenotype except for the truncated Cbn28. The results demonstrate that very minor changes in the peptide sequence may drastically alter antimicrobial activity but that the induction of bacteriocin production is much more tolerant of structural modification, especially at the N terminus.

Lipophilic antimicrobial peptides and proteins occur widely in virtually all types of living systems (1–5), and those produced ribosomally by bacteria are designated as bacteriocins (6). Although the majority of studies on bacteriocins have focused on compounds generated by Gram-negative bacteria (e.g. colicins and microcins), interest in the corresponding peptides from Gram-positive organisms is increasing rapidly (7), especially those from lactic acid bacteria (LAB) (8). The economic importance of these microorganisms and their antimicrobial properties in food and feed production continue to stimulate investigation of LAB bacteriocins as nontoxic food-preserving agents (9).

Carnobacteriocin B2 (CbnB2) is a well characterized, thermostable, cationic bacteriocin that occurs in the culture supernatant of *Carnobacterium piscicola* LV17B, a *Lactobacillus* type organism originally isolated from chill-stored meats (10). CbnB2 targets the cytoplasmic membrane of sensitive cells and causes dissipation of the proton motive force with leakage of intracellular components, possibly through formation of pores (2). Its spectrum of antimicrobial activity includes many LAB as well as strains of potentially pathogenic *Enterococcus* and *Listeria* species (10). A second and unexpected biological role of CbnB2 is to function as an inducer peptide. Addition of the bacteriocin to cultures of *C. piscicola* LV17B that lost the ability to produce bacteriocin upon dilution below 1 × 10^4 cells/ml induces rescue of the Bac− phenotype (11). CbnB2 is ribosomally synthesized as a precursor, precarnobacteriocin B2, which contains 66 amino acids (10). This undergoes post-translational cleavage at a Gly−Gly− site to remove an 18-amino acid leader sequence from the N terminus to yield the mature bacteriocin of 48 amino acids (i.e. CbnB2) that is found in the culture supernatant. The genetic determinant of CbnB2 (cbnB2) is located in a gene cluster present on a 61-kilobase plasmid in *C. piscicola* LV17B (10). This cluster has four other genes required for the wild type Bac− phenotype that, based on sequence homology, function as (i) an ATP binding cassette-type transporter and (ii) an “accessory protein” of the bacterial general secretion pathway-independent secretion system, (iii) a histidine protein kinase and (iv) a response regulator of the bacterial two component signal transduction system (2). A fifth gene in the cluster, cbiB2, encodes an immunity protein (111 amino acids) that protects the producing organism against the antimicrobial effect of carnobacteriocin B2 (13).

CbnB2 has considerable sequence similarity to other bacteriocins produced by LAB, especially near the N terminus, where a characteristic motif of Tyr-Gly-Asn-Gly-Val (YNGV) is highly conserved (10) (see “Discussion”). Because many of these bacteriocins have quite different spectra of antimicrobial activity, the specificity for particular target organisms may be

---

*This work was supported by Natural Sciences and Engineering Research Council Strategic Grant 101231 and an Alberta Agriculture Farming for the Future Research Grant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Harvard Medical School, Dept. of Biological Chemistry and Molecular Pharmacology, LHRBB, 200 Longwood Ave., Boston, MA 02114.

¶ To whom correspondence should be addressed: Dept. of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada. Tel.: 403-492-5475; Fax: 403-492-8231; E-mail: john.vederas@ualberta.ca.

---

1. The abbreviations used are: LAB, lactic acid bacteria; CbnB2, carnobacteriocin B2; HPLC, high performance liquid chromatography; CbnB2P, precarnobacteriocin B2; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; AU, arbitrary units.

2. M. J. Van Belkum, T. Abee, and M. E. Stiles, unpublished data.

3. L. E. N. Quadri, K. L. Roy, J. C. Vederas, and M. E. Stiles, submitted for publication.
determined in part by the much more variable C-terminal region. Access to substantial quantities of specifically mutated LAB bacteriocins of this type would allow detailed examination of structure-activity relationships. The insights gained into the structural requirements governing specificity of action, potency, and role as an inducer of bacteriocin production could serve as a guide to functional requirements for a host of closely related bacteriocins. A recent report on synthetic versions of mesentericin Y105, a 37-amino acid YGNVG bacteriocin, demonstrates that this peptide cannot be truncated at either the C or N terminus without loss of activity (14). In this study, we describe the successful overexpression, production, and purification from Escherichia coli of CbnB2, its precursor precarnobacteriocin B2, and six CbnB2 variants. The results show that certain peptides with reduced or no antimicrobial effect can still act as inducers of bacteriocin production, although one example lacks both antimicrobial and inducing activity. Our study also demonstrates that single amino acid substitution or C-terminal truncation can have a profound effect on the biological activity of the purified peptides.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Determination of Antimicrobial Activity—Carnobacterium and E. coli cultures were grown in APT and Luria-Bertani medium, respectively, as described previously (15, 16). Ampicillin (100 μg ml−1) was added to Luria-Bertani medium for growth and selection of E. coli transformants. The antimicrobial activity of the peptides was determined by the “spot-on-lawn” test (15). Briefly, plates containing approximately 20 ml of APT agar were overlaid with 10 ml of soft APT agar (0.75% agar) inoculated with a 24-h culture of the sensitive indicator strain Carnobacterium divergens LV13 (1% inoculum). Serial 2-fold dilutions of the stock solutions of purified peptides (1 mg ml−1, see below) were prepared in 0.1% trifluoroacetic acid, and up to 20 μl of each stock and its dilutions were spotted (20 μl) onto the bacterial lawn. The plates were kept at 24 °C for approximately 16 h before the presence of inhibition zones was recorded. The results were expressed in arbitrary units (AU) of bacteriocin (1 AU is the minimal amount of peptide required to produce a visible clearing on the lawn of the indicator strain; inhibition was recorded as positive if a distinct clearing was observed; the limit of detection was 0.05 AU μg−1). C. divergens LV13 transformed with plQ4006, a plasmid expressing the carnobacteriocin B2 immunity protein that protects the strain against CbnB2 (13), was also challenged with the purified peptides in the same way as C. divergens LV13. No inhibition of the indicator strain was detected when 0.1% trifluoroacetic acid alone was used in the assay.

DNA Manipulation and DNA Sequencing Analysis—Restriction digests, 5′-labeling of probes with [γ-32P]ATP, colony blot hybridization, and translational frame size: Scientific and Technical Services, University of Guelph, Ontario, standard procedures (16). Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Boehringer Mannheim. E. coli JM107 was used as host for all cloning experiments (16). Transformation of E. coli competent cells and selection and screening of transformants were done by established methods (16). DNA was sequenced bidirectionally and analyzed with an Applied Biosystems 373A DNA sequencer using fluorescent dye chain terminators. The reactions were primed with M13/pUC sequencing primer and were analyzed with an Applied Biosystems model 470A with on-line phenylthiohydantoin derivative identification by reversed-phase HPLC (Applied Biosystems model 120A). The mass spectra of the purified peptides were done by direct injection of their solutions (50% aqueous acetonitrile, 0.1% trifluoroacetic acid) using a VG Quattro multiple quadrupole instrument with an electrospray ionization source (Fisons, Manchester, United Kingdom).

Bacteriocin Induction with CbnB2 and Its Variants—Induction experiments were done as described previously with the following modifications (11). C. piscicola LV17B cultures with suppressed bacteriocin production (Bac− cultures) were obtained by subculturing bacteriocin-producing cultures (Bac+ cultures) using an inoculum below 1 × 104 cells/ml of broth. Before subculture of the Bac− cell, the medium was supplemented with CbnB2 or its antimicrobically active variants to give final concentrations of 5, 15, and 30 AU ml−1, respectively. For the

Protein Engineering of Carnobacteriocin B2

Cycles (denaturing step, 95 °C, 30 s; annealing step, 52 °C, 45 s; extension step, 72 °C, 1.5 min). The PCR products were separated using agarose gel electrophoresis and purified by standard procedures (16). Construction of Recombinant Protein Genes—The amplified fragments containing the coding sequence for the precursor of carnobacteriocin B2 or the mature peptide of the E. coli were digested with XbaI and inserted into the expression vector pMAL-c linearized with StuI and XbaI (New England Biolabs). The insertion of such fragments in pMAL-c created a fusion in the correct translational reading frame between the maltose-binding protein gene (malE) of E. coli located in pMAL-c and the carnobacteriocin B2 coding region. Transformants containing the correct inserts were identified by colony hybridization using the primer LQRM as a probe. The inserts of several clones were sequenced.

Screening for Overproduction and Purification of Fusion Proteins—For screening and large scale production of fusion proteins, E. coli strains were grown in Rich broth containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 2 g of glucose, and 2 ml of ampicillin solution (50 mg ml−1) per liter, or in complete minimal medium containing 6 g of Na2HPO4, 3 g of KH2PO4, 0.5 g of NaCl, 1 g of NH4Cl, 2 ml of 1 M MgSO4, 20 ml of 20% D-glucose, 1 ml of 1 M thiamine, and 2 ml of ampicillin solution (50 mg ml−1) per liter. Production of recombinant proteins was induced with isoprropyl-1-thio-β-D-galactopyranoside (0.3 mm) when the cultures reached an optical density at 600 nm of 0.5. After induction, the cultures were incubated for 3 h before they were harvested by centrifugation. The presence of overexpressed recombinant proteins in E. coli was screened by SDS, 12% polyacrylamide gel electrophoresis (PAGE). Total cell proteins from 100 μl of an isopropyl-1-thio-β-D-galactopyranoside-induced culture were loaded in SDS-PAGE. In the large scale purification the induced cells (1 L of culture) were harvested and resuspended in column buffer (100 ml, containing 20 mM Triis-Cit, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM sodium azide, pH 7.4). After this step, all manipulations were done at 4 °C. Cells were lysed in a French pressure cell at 1300 p.s.i. and sonicated three times for 2 min. The lysates were centrifuged at 7000 × g for 20 min, and the resulting supernatant was diluted to 700 ml with column buffer. The lysates were loaded onto a 25-ml amylose resin column (flow rate, 0.5 ml min−1). After the column was washed with 12 to 15 volumes of column buffer, the recombinant proteins were eluted with the same buffer containing 10 mM maltose. Fractions containing the recombinant proteins were dialyzed against Milli-Q water, lyophilized, and stored at −20 °C.

Cleavage of Fusion Proteins and Purification of Bacteriocins—The cleavage of the fusion proteins with Factor Xa was done as recommended by New England Biolabs (except as noted). The initial protein concentration was From 1 to 2 mg ml−1. The cleavage time was from 7 to 9 h except for the fusion proteins bearing CbnS33 and CbnB2P, which were cut for 10 and 12 h, respectively. The mature bacteriocin B2, the precursor peptide (precarnobacteriocin B2), and the carnobacteriocin variants were purified by reversed-phase HPLC using a C4 Vydac column (10 × 250 mm, 10-μm particle size, 300 Å pore size, Scientific and Technical Services, University of Guelph, Canada) and gradient elution (flow rate, 2.5 ml min−1, monitored at 218 nm). Solvent A was 0.1% trifluoroacetic acid in H2O, and Solvent B was 70% acetonitrile/H2O with 0.1% trifluoroacetic acid. Gradients were 30–70% B in 25 min followed by 70–90% B in 5 min. Purified peptides were lyophilized, resuspended in 0.1% trifluoroacetic acid to a concentration of 1 mg ml−1, and stored at −20 °C. Protein concentration was determined using Bio-Rad Protein Assay or by HPLC (monitoring at 218 nm) using carnobacteriocin B2 as a standard.

N-terminal Sequence Analyses and Mass Spectrometry—Purified peptides were analyzed by the Alberta Peptide Institute (University of Alberta). The N-terminal amino acid sequences were obtained using 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 spectra of the purified peptides were done by direct injection of their solutions (50% aqueous acetonitrile, 0.1% trifluoroacetic acid) using a VG Quattro multiple quadrupole instrument with an electrospray ionization source (Fisons, Manchester, United Kingdom).

Bacteriocin Induction with CbnB2 and Its Variants—Induction experiments were done as described previously with the following modifications (11). C. piscicola LV17B cultures with suppressed bacteriocin production (Bac− cultures) were obtained by subculturing bacteriocin-producing cultures (Bac+ cultures) using an inoculum below 1 × 104 cells/ml of broth. Before subculture of the Bac− cell, the medium was supplemented with CbnB2 or its antimicrobically active variants to give final concentrations of 5, 15, and 30 AU ml−1, respectively.
inactive peptides (Cbn28, CbnS33, and CbnB2P) induction experiments employed concentrations of 2.5 and 7.5 μg ml⁻¹ of medium. Cbn28 was also tested at 30 μg ml⁻¹. Bacteriocin activity present in the culture supernatant was evaluated when the cultures reached the stationary phase of growth. The inhibitory activity of the heat-treated supernatant (65 °C for 30 min) was assayed by the spot-on-lawn technique using a lawn of C. divergens LV13. Background activity from the bacteriocins added to induce production was taken into account in determining induction at high concentration. Bac⁺ supernatant was included as a positive control.

RESULTS

Construction of Recombinant Proteins and Identification of Carnobacteriocin B2 Mutants—The PCR-amplified DNA fragments encoding the precursor (precarnobacteriocin B2) or the mature bacteriocin (carnobacteriocin B2) were ligated to the maltose-binding protein gene (malE) to create the translational fusions. To confirm the inducibility of the expression system, several clones that contained inserts of the expected size were screened for the production of the recombinant proteins. Analysis of the overexpressed proteins containing precarnobacteriocin B2 by SDS-PAGE indicated that all of the clones produced a protein of the expected size. One of these clones, pLQP, was sequenced to verify that there were no errors in the nucleotide sequence. This was then used for the large scale purification of precarnobacteriocin B2. In the case of the clones expressing the fusion protein containing carnobacteriocin B2, smaller products were observed in a few cases (data not shown). Because the presence of smaller recombinant proteins could indicate an unexpectedly high frequency of mistakes during the PCR amplification step, several clones were sequenced. Analysis indicated that the majority of the clones contained the correct nucleotide sequence. One of these, pLQM, was used for the large scale purification of carnobacteriocin B2. Analysis of the DNA sequence allowed the identification of six clones containing mutations in the coding region for carnobacteriocin B2. Five of these mutants had only one amino acid substitution (pLQF3, pLQS33, pLQI34, pLQI37, and pLQG46), and another one had a frameshift mutation that replaced the last 20 amino acid residues of carnobacteriocin B2 with 5 other residues from a different coding frame (pLQ28). These clones were used for the purification of the carnobacteriocin B2 variants. The amino acid sequences of these variants and those of precarnobacteriocin B2 and carnobacteriocin B2 are shown in Fig. 1.

Purification and Characterization of Carnobacteriocin B2, Precarnobacteriocin B2, and Bacteriocin Variants—Precarnobacteriocin B2 (CbnB2P), CbnB2, and the six mutants (CbnF3, CbnS33, CbnI34, CbnI37, CbnG46, and Cbn28) (Fig. 1) were expressed in E. coli as recombinant proteins fused to the maltose-binding protein encoded on pMAL-c. After purification on an amylose column, between 20 and 50 mg of the fusion proteins containing CbnB2P, CbnB2, CbnF3, CbnI34, CbnI37, CbnG46, or Cbn28 were recovered per liter of fermentation culture. The purified recombinant proteins represent approximately 10 to 25% of the total cellular protein. In the case of CbnS33, only 3.3 mg of the corresponding fusion protein could be purified from 1 L of culture, representing approximately 1.6% of the total cellular protein.

The presence of a recognition sequence for Factor Xa allowed the cleavage of the recombinant proteins to release the carnobacteriocin-related peptides without any extra amino acids at the N terminus. During this reaction only 12 to 37% of the substrate was cleaved, as indicated by the intensity of the bands visualized in SDS-PAGE and the peak areas in HPLC separation (data not shown). More prolonged exposure to Factor Xa was deleterious and led to side products, presumably because of nonspecific fission of other peptide bonds. After the cleavage reaction, the carnobacteriocin-related peptides were separated and purified to homogeneity by reversed-phase HPLC. The retention times of the maltose-binding protein and the undigested fusion proteins were 28 and 27 min, respectively. Under identical conditions, the retention times of CbnB2, CbnB2P, CbnF3, CbnS33, CbnI34, CbnI37, CbnG46, and Cbn28 were 19.5, 19.1, 19.1, 13.9, 19.8, 19.5, 18.4, and 16.7 min, respectively. The approximate amounts of bacteriocins isolated in milligrams per liter of culture are: CbnB2, 1.40; CbnB2P, 0.24; CbnF3, 1.40; CbnS33, 0.04; CbnI34, 0.56; CbnI37, 1.40; CbnG46, 0.73; and Cbn28, 0.74. The first 5 amino acids of the purified peptides were determined by Edman degradation in each case. The amino acid sequences were the same as those predicted from the DNA sequences, thereby verifying that the correct cleavage had occurred with Factor Xa at the N terminus of the carnobacteriocin-related peptides. To ascertain that the peptides were fully translated and to determine whether they were posttranslationally modified in E. coli, the molecular weight of each was determined by electrospray mass spectrometry. The calculated and the predicted molecular weight of all peptides are in agreement (Table I). CbnB2 purified from C. piscicola contains its cysteine residues in a reduced state. For the peptides produced in E. coli, comparison between the calculated molecular weight (with reduced cysteine residues) and the molecular mass of each peptide determined by mass spectrometry indicates that cysteine residues are in a reduced state (Table I). For CbnI37, it is not possible to determine the redox state of the cysteine residues due to the error limits in the molecular mass determination.

The antimicrobial activities of the purified carnobacteriocin-related peptides and of the fusion proteins were determined against the sensitive indicator strain C. divergens LV13. The specific activities of CbnB2 (parent bacteriocin), CbnI34, CbnI37, and CbnG46 were 50 AU μg⁻¹ for each peptide. The prebacteriocin CbnB2P and CbnF3 had specific activities of 0.4 and 7 AU μg⁻¹, respectively. No activity was detected for Cbn28 and CbnS33 or for any of the fusion proteins under the
condition of the assay, which has a limit of detection of 0.05 AU μg⁻¹. C. divergens LV13 transformed with pLQ400i is immune to the bacteriocin CbnB2 produced from E. coli, as previously reported for the corresponding compound isolated from C. piscicola LV17B (13). The peptides CbnI34, CbnI37, CbnG46, and CbnF3 were not active against this strain of C. divergens LV13 expressing carnobacteriocin B2 immunity protein. This indicates that the immunity protein protects the strain from the antimicrobial activity of these carnobacteriocin B2 variants.

The ability of the purified peptides to function as inducers of bacteriocin production was determined. Induction was marginal (50 AU ml⁻¹ of culture supernatant) when CbnB2, CbnI34, CbnI37, and CbnG46 were added at a final concentration of approximately 0.1 μg ml⁻¹ (5 AU ml⁻¹) or when CbnF3 was added to a final concentration of 0.7 μg ml⁻¹ (5 AU ml⁻¹). However, bacteriocin production was induced to the level of the control (Bac⁺ culture or Bac⁺ culture induced with 2% supernatant of Bac⁺ culture) when the peptides were added to a final concentration of 0.3 μg ml⁻¹ (15 AU ml⁻¹) or 2.2 μg ml⁻¹ (15 AU ml⁻¹) for CbnF3. The bacteriocin precursor CbnB2P and CbnS33 induced bacteriocin production at a final concentration of 2.5 and 7.5 μg ml⁻¹, respectively. However, Cbn28, the C-terminal truncated peptide, did not induce bacteriocin production at a final concentration of up to 30 μg ml⁻¹.

**DISCUSSION**

We have overexpressed the mature carnobacteriocin B2, six engineered variants, and the bacteriocin precursor precarnobacteriocin B2 as fusions with maltose-binding protein in E. coli and have specifically cleaved these to release the parent peptides. Because some of the purified peptides have little or no antimicrobial activity, direct detection of these target compounds in a complex fermentation mixture would be difficult and would have made their purification particularly troublesome. Their expression as maltose-binding protein fusions greatly facilitates their purification through affinity chromatography on amylose resin and affords substantial quantities of the proteins (20–50 mg liter⁻¹ except for the CbnS33 fusion). The cleavage reaction with Factor Xa also proceeds reasonably to generate useful quantities of the parent peptides. Typically, the yield of HPLC-purified peptide is about 1.4 to 0.6 mg liter⁻¹ except for CbnB2P and CbnS33. Mass spectral analyses indicate that all of the cysteine residues are in the reduced state with the possible exception of CbnI37, where deviations in measurement preclude definitive assignment of oxidation state. The wild type mature bacteriocin, CbnB2, produced in this manner possesses characteristics (HPLC retention time, mass spectra, reduced cysteine residues, N-terminal amino acid sequence) identical to those of the compound purified from the natural host, C. piscicola (10), and also displays the expected specific antimicrobial activity and inducing properties for bacteriocin production. The fusion proteins have no detectable antimicrobial activity, which is not surprising because the maltose-binding protein part imposes a huge size (42,700 daltons) and polarity difference relative to the attached hydrophobic bacteriocin. It has the potential to alter the preferred three-dimensional conformation and accessible surface of the parent peptide.

Examination of the sequences of known LAB bacteriocins having the YGNGV motif (10) and consideration of the specificities (antimicrobial spectra) of closely related members of this group suggest that occurrence of at least one key molecular recognition event is essential for bacteriocin antimicrobial activity (17, 18). This probably involves binding to an unidentified protein receptor in the bacterial membrane because it is unlikely that simple self-association and pore formation can account for the specificity of antibacterial action. Occurrence of such a receptor has been suggested in studies on the mode of action of pediocin PA-1, a structurally related bacteriocin of this class (19). The immunity protein that, when expressed within the cell, protects sensitive organisms from carnobacteriocin B2 is probably also involved in a molecular recognition event (perhaps with the same receptor), although it does not significantly bind to this bacteriocin directly and affords no protection if co-administered with CbnB2 from outside the sensitive cell (13). The variants of CbnB2 produced in the present study offer interesting insights into structure-activity relationships. The presence of the 18-amino acid leader peptide in the purified precursor, CbnB2P, reduces the antimicrobial activity by a factor of 125. This may be due to alteration of the preferred three-dimensional conformation and/or exposed surfaces available for binding to cellular constituents. The residual activity of this intracellular bacteriocin precursor, although low, may necessitate the observed co-expression of immunity protein in wild type C. piscicola that is encoded on the same operon. Reduction of antimicrobial properties for precursors of other types of bacteriocins has previously been suggested (20).

The other purified bacteriocin variants with reduced or no activity are CbnF3 (Tyr⁴ → Phe), CbnS33 (Phe³³ → Ser), and Cbn28, which has a frameshift mutation that replaces the last 20 amino acid residues of CbnB2 by 5 other residues from a different coding frame. The lack of activity of Cbn28 is not surprising because it represents a major structural change from the parent bacteriocin. However, it is interesting that although the only difference between the wild type carnobacteriocin B2 and CbnF3 is replacement of a hydroxyl group by hydrogen, the antimicrobial specific activity is reduced 7-fold. This small change in a peptide of 48 amino acids with a mole-
cular weight of 4967 demonstrates that the tyrosine residue in the YGN GV conserved motif is important for full biological activity. The effect of this amino acid substitution is particularly relevant because it could potentially be extended to at least six other natural bacteriocins that contain this sequence motif (Fig. 2). Recent studies on synthetic analogs of mesentericin Y105 support the hypothesis that highly conserved residues 1–14 of this 37-amino acid bacteriocin form part of a recognition sequence for a membrane-bound receptor but also indicate that the entire structure, including the N and C termini, plays a critical role in antimicrobial activity (14). Removal of Trp37 or the N-terminal residues 1–3 from mesentericin reduces the inhibitory activity of mesentericin Y105 by a factor of 10^4 or more. In the case of carnobacteriocin B2, the L-Phe^33 is also critical for the antimicrobial activity. Its replacement by the polar amino acid L-Ser in CbnS33 abolishes the activity of the peptide entirely. This amino acid substitution also decreases the HPLC retention time (13.9 versus 19.5 min for CbnB2) on a reversed-phase column (C8), indicating that the change significantly reduces the overall hydrophobicity of this variant.

In contrast, three other variants with single amino acid substitutions are fully active: CbnI34 (Val^34 → Ile), CbnI37 (Val^37 → Ile), and CbnG46 (Arg^66 → Gly). Because the isoleucine for valine changes are minimal in terms of hydrophobicity and structural variation (i.e., addition of a methyl group at the terminus of the valine residue), it is not surprising that the CbnI34 and CbnI37 bacteriocin mutants are fully active. The conservative differences between the YGNV bacteriocins leucoin A (21, 22) and mesentericin Y105 (17, 18), namely replacement of Phel^22 by Ala and substitution of Val^26 by Ile in a 37-amino acid peptide, result in only slight variation in the levels of activity against identical organisms (14). The much more drastic alteration of Arg^66 in CbnB2 to Gly in CbnG46 did not affect the antimicrobial activity of the peptide, and this indicates that the side chain of this residue is not critical.

In order to examine influences of structural variation in the bacteriocin on the efficacy of the immunity protein that protects against the action of the parent CbnB2 (13), the activity of the peptides CbnI34, CbnI37, CbnG46, and CbnF3 was tested against a previously sensitive indicator strain (C. divergens LV13) that was modified to express the immunity protein. The resulting strain was immune to these peptides as well as to the naturally occurring CbnB2, indicating that the changes introduced in their amino acid sequences did not compromise the mechanism of immunity. This result is in accord with the previous observation that the isolated immunity protein does not bind significantly to the parent bacteriocin CbnB2 in vitro (13). Genes for related immunity proteins have been found in other LAB bacteriocin-producing systems (17, 22–25).

Carnobacteriocin B2 not only acts as an antimicrobial peptide, it also induces bacteriocin production, probably by a signal transduction mechanism. In order to examine the influence of structural variation in carnobacteriocin B2 on the latter effect, we investigated the ability of CbnB2, CbnB2P, CbnF3, CbnS33, CbnI34, CbnI37, CbnG46, and Cbn28 to induce bacteriocin production. All of the peptides except Cbn28 induced Bac^- cultures to reestablish their Bac^+ phenotype. Because CbnS33 is not active and still functions as an inducer, it is possible to conclude that the antimicrobial activity of the bacteriocin is not required for its function as inducer. Furthermore, the fact that Cbn28 fails to induce, despite having the N-terminal 28 amino acids present in CbnB2, indicates that the C-terminal portion of the peptide is essential for this function. The ability of the precursor CbnB2P, which has an 18-amino acid extension at the N terminus of CbnB2, to induce bacteriocin production suggests that a free N terminus in the bacteriocin is not required. More detailed studies on the signal transduction and regulation pathway involving carnobacteriocin B2 are in progress.

The availability of the current strategy for effective generation of bacteriocins and their variants has allowed facile isotopic labeling of CbnB2 with ^15N in E. coli (data not shown) for ongoing NMR studies to determine three-dimensional conformation in lipophilic environments. Although such labeling of parent bacteriocins can be done with wild-type LAB organisms using a multistage procedure involving preparation of labeled cyano bacterial peptone (26), the present method for generation of bacteriocins and their precursors affords much easier access to substantial quantities of labeled peptides. The expression of bacteriocin variants as maltose-binding protein fusions will also allow production of peptides that cannot be directly engineered into a natural host. For example, variants with amino acid substitutions that prevent the secretion or make them toxic to the producer could be expressed and purified as fusions in E. coli. In addition to providing probes for the biological machinery involved in bacteriocin production and mode of action, the present methodology could assist in the design of new antimicrobial agents with improved activity spectra and properties for commercial food applications. Studies on these aspects as well as on the three-dimensional structure of bacteriocins are ongoing.

Acknowledgments—We are indebted to Dr. Gordon Alton for extensive assistance with mass spectrometry and to Prof. Kenneth L. Roy for helpful discussions.

REFERENCES
1. Rao, A. G. (1995) Mol. Plant-Microbe Interact. 8, 6–13
2. Martin, E., Ganz, T., and Lethrer, R. I. (1995) J. Leukocyte Biol. 58, 128–136
3. Broekaert, W. F., Terras, P., Frier, E., Gammeur, B. P. A., and Osborn R. W. (1995) Plant Physiol. 105, 1533–1538
4. Braun, V., Pilsl, H., and Gross, P. (1994) Arch. Microbiol. 161, 199–206
5. Baquero, F., and Moreno, F. (1984) FEMS Microbiol. Lett. 23, 117–124
6. Tagg, J. R., Dujani, A. S., and Wannamaker, L. W. (1976) Bacteriol. Rev. 40, 772–756
7. Jack, R. W., Tagg, J. R., and Ray, B. (1995) Microbiol. Rev. 59, 191–200
8. Kaadenhammer, T. R. (1995) FEMS Microbiol. Rev. 12, 381–395
9. Vandenbergh, P. A. (1993) FEMS Microbiol. Lett. 12, 221–228
10. Quadri, L. E. N., Sailer, M., Roy, K. L., Vederas, J. C., and Stiles, M. E. (1994) J. Biol. Chem. 269, 12204–12211
11. Sancier, L., Poon, A., and Stiles, M. E. (1995) J. Appl. Bacteriol. 78, 684–690
12. Bukhittiyaruva, M., Yang, R., and Ray, B. (1994) Appl. Environ. Microbiol. 60, 3405–3408
13. Quadri, L. E. N., Sailer, M., Terezieczik, M. R., Roy, K. L., Vederas, J. C., and Stiles, M. E. (1995) J. Bacteriol. 177, 1144–1151
14. Fleury, Y., Dayem, M. A., Montagne, J. J., Chabosseau, E., Le Caer, J. P., Nicolas, P., and Defour, A. (1996) J. Biol. Chem. 271, 14421–14429
15. Ahn, C., and Stiles, M. E. (1995) Appl. Environ. Microbiol. 61, 2505–2510
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Fremaux, C., Hechard, Y., and Cenatiempo, Y. (1995) Microbiology 141, 1637–1645
18. Hechard, Y., Dériard, B., Letellier, F., and Cenatiempo, Y. (1992) J. Gen. Microbiol. 138, 2725–2731
19. Chikindas, M. L., Garcia, B., Jin, M. J., Driessen, A. J. M., Ledeboer, A. M., Nissen-Meyer, J., Nes, I. F., Abe, T., Konings, W. N., and Venema, G. (1993) Appl. Environ. Microbiol. 59, 3577–3584
20. Hayar, L. S., Diep, H. H., and Nes, I. F. (1995) Mol. Microbiol. 16, 229–240
21. Hastings, J. W., Sailer, M., Johnston, K. R., and Venema, G. J. (1993) J. Bacteriol. 173, 7491–7500
22. Van Belkum, M., and Stiles, M. (1995) Appl. Environ. Microbiol. 61, 3573–3579
23. Holck, A., Axellsson, L., Birkeland, S.-E., Aukrust, T., and Blom, H. (1992) J. Gen. Microbiol. 138, 2715–2720
24. Axellsson, L., and Holck, A. (1993) J. Bacteriol. 177, 2125–2137
25. Venema, K., Kok, J., Marugg, J. D., Toonen, M. Y., Ledeboer, A. M., Venema, G., and Chikindas, M. L. (1995) Mol. Microbiol. 17, 515–522
26. Sailer, M., Helma, G. L., Henkel, T., Nienzaura, W. F., Stiles, M. E., and Vederas, J. C. (1993) Biochemistry 32, 310–318
27. Tichacek, P. S., Vogel, R. F., and Hammes, W. P. (1994) Microbiology 140, 361–367
28. Holck, A., Axellsson, L., Huhne, K., and Krockel, L. (1994) FEMS Microbiol. Lett. 115, 143–150
29. Marugg, J. D., Gonzalez, C. F., Kunka, B. S., Ledeboer, A. M., Pucci, M. J., Toonen, M. Y., Walker, S. A., Zoetmulder, L. C. M., and Vandenbergh, P. A. (1992) Appl. Environ. Microbiol. 58, 2360–2367