Microbiology (2000), **146**, 621–631 Printed in Great Britain

Characterization of the genetic locus responsible for production and immunity of carnobacteriocin A: the immunity gene confers cross-protection to enterocin B

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Carnobacteriocin A (CbnA) is a regulated bacteriocin produced by *Carnobacterium piscicola* LV17A that is encoded on a 72 kb plasmid. A 10·0 kb fragment from this plasmid that contained information necessary for bacteriocin production and immunity was cloned and sequenced. Genetic analysis showed the presence of the previously sequenced structural gene for CbnA, as well as genes encoding proteins homologous to dedicated bacteriocin transport proteins and proteins of three-component signal transduction systems. The induction factor (CbnX) was chemically synthesized and induced CbnA production at 10^{-11} M or higher in a *C. piscicola* LV17A culture that had lost the ability to produce bacteriocin as a result of dilution. The gene *cbiA* for the immunity protein is not located in typical close proximity to the structural gene for CbnA and is encoded in the opposite orientation. CbiA has homology with EniB, the immunity protein for enterocin B that is also encoded in the opposite orientation to the bacteriocin gene. CbiA and EniB cross-protected against the corresponding bacteriocins.

Keywords: bacteriocin, lactic acid bacteria, *Carnobacterium piscicola*, carnobacteriocin A, enterocin B

INTRODUCTION

Lactic acid bacteria (LAB) produce a wide array of class II bacteriocins that have been classified by Klaenhammer (1993) and Nes *et al*. (1996). These are small (4–6 kDa), heat-stable peptides that are ribosomally synthesized as inactive prebacteriocins, with either an N-terminal leader peptide that is post-translationally cleaved at a conserved Gly-Gly site, or an N-terminal signal peptide. Class IIa bacteriocins are '*Listeria*-active' and contain a -YGNGVXC- amino acid motif near the N-terminus of the mature peptide. Class IIb bacteriocins consist of two peptides, for example brochocin-C (McCormick *et al*., 1998) and lactococcin G (Nissen Meyer *et al*., 1992); both peptides are required for activity. Class IIc, according to Klaenhammer (1993), contains thiol-activated bacteriocins, whereas according to Nes *et al*. (1996), class IIc bacteriocins are those that depend on the general secretory pathway for export of the bacteriocin from the cell. Production of class II bacteriocins, in the absence of regulatory genes, depends on at least four genes requiring 4–5 kb of DNA, i.e. the gene(s) for the prebacteriocin, an immunity gene, and genes for a dedicated ATP-binding cassette (ABC) transporter protein and an accessory protein (Nes *et al*., 1996). The bacteriocin immunity gene is usually located on the same transcription unit next to, and downstream of, the bacteriocin structural gene, and it is expressed

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Abbreviations: ABC, ATP-binding cassette; Ap, ampicillin; ATCC, American Type Culture Collection; Bac, bacteriocin production; CbiA, carnobacteriocin A immunity protein; Cbn, carnobacteriocin; Em, erythromycin; DSM, Deutsche Sammlung von Mikroorganismen; DvnA, divergicin A; EniB, enterocin B immunity protein; EntB, enterocin B; LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AF207838

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with the bacteriocin gene (Nes *et al*., 1996). The immunity protein of class II bacteriocins protects the cell from its own bacteriocin. Despite the similarities observed for many class II bacteriocins, their immunity proteins share limited or no homology (Allison & Klaenhammer, 1996; Aymerich *et al*., 1996; Nes *et al*., 1996; Eijsink *et al*., 1998).

Production of several class IIa bacteriocins is regulated by a three-component regulatory system that is homologous to signal transduction systems in bacteria (Nes *et al*., 1996). The components of this regulatory system consist of an induction factor, a histidine protein kinase and a response regulator (Nes *et al*., 1996). Production of the plantaricins S, JK and EF by *Lactobacillus plantarum* strains (Diep *et al*., 1995, 1996; Anderssen *et al*., 1998; Stephens *et al*., 1998), sakacins A and P by *Lactobacillus sakei* (Axelsson & Holck, 1995; Hühne *et al*., 1996; Brurberg *et al*., 1997), carnobacteriocin B2 by *Carnobacterium piscicola* LV17B (Quadri *et al*., 1997), and enterocins A and B by *Enterococcus faecium* strains (Nilsen *et al*., 1998; O'Keeffe *et al*., 1999), are regulated by three-component regulatory systems.

C. piscicola LV17 produces three carnobacteriocins: A, BM1 and B2. The structural gene for carnobacteriocin BM1 (CbnBM1) is located on the chromosome and those for carnobacteriocins A (CbnA) and B2 (CbnB2) are located on 72 kb and 61 kb plasmids, respectively (Quadri *et al*., 1994; Worobo *et al*., 1994). By curing and plasmid mobilization the plasmids were separated and introduced into the plasmidless host strain (LV17C) as LV17A and LV17B, respectively (Ahn & Stiles, 1992). The immunity genes for CbnBM1 and CbnB2 are located in an operon with the respective prebacteriocin structural genes (Quadri *et al*., 1994), but an immunity gene was not located in the same relative position for CbnA (Worobo *et al*., 1994). This was similar to the case of enterocin B (EntB) production by *Ent. faecium* BFE 900, where the immunity gene was not located within the same operon (Franz *et al*., 1999).

We previously purified CbnA and localized the gene encoding precarnobacteriocin A on a 1·4 kb cloned fragment (Worobo *et al*., 1994). In this study we describe the nucleotide sequence and genetic arrangement of the region downstream of the CbnA structural gene, and its relationship to immunity, dedicated bacteriocin transport and regulation of CbnA production. We also describe the fusion of the DNA encoding the signal peptide of the divergicin A gene to the part of the gene encoding mature CbnA, followed by the gene for CbiA. This study also reports cross-immunity between the CbnA and EntB immunity genes in the respective CbnA and EntB bacteriocin systems.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown in Luria–Bertani broth (LB; BBL, Becton Dickinson) at 37 °C on a rotary shaker at 250 r.p.m. Carnobacteria were grown in APT broth (All Purpose Tween; Difco). Other LAB were grown in Lactobacilli MRS broth (Difco) at 25 °C without agitation. Stock cultures were prepared in the same media with 15 % (v/v) glycerol and stored at −80 °C. Antibiotics were added as selective agents when appropriate: erythromycin (200 μg ml^{-1}) and ampicillin (150 μg ml^{-1}) for *E. coli*, erythromycin (5 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}) for LAB.

Bacteriocin activity assays. Bacteriocin activity was quantified by the critical dilution method (Franz *et al*., 1999), using heat-treated (boiling water bath for 6 min) cell-free supernatant of the producer culture. Indicator bacteria from an overnight culture were inoculated (1 %) into APT soft agar (0·75 % agar). *C. piscicola* LV17C was used as a sensitive indicator strain for CbnA, while *L. sakei* DSM 20017 was used as a sensitive indicator for both CbnA and EntB. Immunity was tested by deferred inhibition assay (Ahn & Stiles, 1990) with *C. piscicola* LV17A or the EntB producer *Enterococcus faecalis* ATCC 19433 containing plasmid pCMAP03 (Franz *et al*., 1999) as producer strains, and strains containing immunity genes as indicators.

DNA manipulation, cloning and transformation. Large- and small-scale plasmid preparations from *E. coli* (Sambrook *et al*., 1989) and carnobacteria (Worobo *et al*., 1994; van Belkum & Stiles, 1995) were done by established techniques. Restriction endonucleases, T4 DNA ligase and Klenow enzyme were used as recommended by the suppliers (New England Biolabs; Promega). DNA manipulations and cloning were done as described by Sambrook *et al*. (1989). Competent cells of *E. coli* were prepared and transformed according to the one-step method of Chung *et al*. (1989). Recombinant pMG36e plasmids were first transformed into *E. coli* MH1 before being transformed into LAB. Transformation of carnobacteria by electroporation was done by established methods (Worobo *et al*., 1995) and *L. sakei* DSM 20017 was transformed by the method of Berthier *et al*. (1996).

DNA sequence analysis. The nucleotide sequence was determined bidirectionally by *Taq* DyeDeoxy Cycle sequencing (Departments of Biochemistry or Biological Sciences, University of Alberta) on an Applied Biosystems 373A sequencer with stepwise deletion derivatives of cloned DNA fragments made with the Erase-a-Base system (Promega). A primer-walking strategy was used to complete small gaps in the nucleotide sequence. Synthetic oligonucleotides were made with an Applied Biosystems 391 PCR-Mate DNA synthesizer (Department of Biological Sciences, University of Alberta). Analysis of the nucleotide sequence was done with the DNA Strider (version 1.2) program. A search for homology of the predicted amino acid sequences was done with the BLAST network service at the National Center for Biotechnology Information (NCBI). Homology comparisons and calculations were done with the DNASTAR program.

Localization of the gene required for carnobacteriocin A immunity. A 10·0 kb *Pst*I fragment from pCP49 was cloned into pCaT (Jewell & Collins-Thompson, 1989), resulting in plasmid pRW01 (Table 1). To localize the CbnA immunity gene, fragments of the 10·0 kb insert in pCAT were cloned into pMG36e and certain ORFs were disrupted by using internal restriction enzyme sites. First, a 5·4 kb *Xba*I–*Pst*I fragment was excised from pRW01, blunt-ended and cloned into the *Sma*I site of the lactococcal shuttle vector pMG36e (van de Guchte *et al*., 1989) with the CbnA structural gene in the same orientation as the P32 promoter to yield plasmid pCF01 (Fig. 1). A 3·1 kb *Pst*I–*Sph*I fragment was excised from pCF01 and cloned into pMG36e resulting in plasmid pCF02. Plasmid pCF03 was created by cutting pCF02 with *Pst*I (located in the

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Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics*	Reference or source
Strains		
Lactobacillus sakei DSM 20017	EntB^s CbnA^s	DSM
Enterococcus faecium BFE 900	*entB*$^+$ *eniB*$^+$ *entA*$^+$, plasmidless	Franz *et al.* (1999)
Carnobacterium piscicola LV17A	*cbnA*$^+$ *cbi*A$^+$, containing pCP49	Ahn & Stiles (1990); Worobo *et al.* (1994); this study
C. piscicola LV17C	CbnA^s, plasmidless	Ahn & Stiles (1990)
Escherichia coli DH5α	F$^-$ *endA1 hsdR17* (r_k^- m_k^+) *supE44* λ^- *thi-1 recA1 gyrA96 relA1* Δ(*argF–lacZYA*) U169 ϕ80d*lacZ* ΔM15	BRL Life Technologies
E. coli MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	Casadaban & Cohen (1980)
Plasmids		
pUC118	*lacZ′* Ap^r, 3·2 kb	Vieira & Messing (1982)
pMG36e	Expression vector, Em^r, 3·6 kb	van de Guchte *et al.* (1989)
pCP49	72 kb plasmid from *C. piscicola* LV17, *cbnA*$^+$ *cbiA*$^+$	Worobo *et al.* (1994)
pRW19e	pMG36e derivative containing divergicin A structural and immunity genes, Em^r, 4·1 kb	McCormick *et al.* (1996, 1998)
pCaT	Cm^r, 8·5 kb	Jewell & Collins-Thompson (1989)
pCMAP03	pRW19e containing 200 bp *Hin*dIII–*Kpn*I PCR product insert that creates the *dvnA* signal peptide::*entB* gene fusion	Franz *et al.* (1999)
pCMAP05	pMG36e containing 197 bp *Xba*I–*Hin*dIII PCR product of *eniB*	Franz *et al.* (1999)
pRW01	pCaT containing 10·0 kb *Pst*I–*Pst*I fragment from pCP49	Worobo (1996)
pCF01	pMG36e containing 5·4 kb *Xba*I–*Pst*I fragment from pRW01	This study
pCF02	pMG36e containing 3·1 kb *Pst*I–*Sph*I fragment from pCF01	This study
pCF03	pMG36e containing 1·9 kb *Bsp*MI–*Sph*I fragment from pCF02	This study
pCF04	pMG36e containing 1·4 kb *Bam*HI–*Sph*I fragment from pCF02	This study
pCF05	pMG36e containing 751 bp *Cla*I–*Sph*I fragment from pCF02	This study
pCF06	pMG36e containing 721 bp *Bam*HI–*Cla*I fragment from pCF02	This study

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Table 1 *(cont.)*

Bacterial strain or plasmid	Relevant characteristics*	Reference or source
pCF07	pMG36e containing 400 bp *Bam*HI–*Hin*dIII fragment from pCF02	This study
pCF08	pMG36e containing 231 bp *Xba*I–*Kpn*I PCR product insert of *cbiA*	This study
pCF09	pRW19e containing 218 bp *Hin*dIII–*Kpn*I PCR product insert that creates the *dvnA* signal peptide::*cbnA* gene fusion	This study
pCF10	pRW19e containing 200 bp *dvnA* signal peptide::*entB* PCR fusion product and 197 bp *eniB* PCR product	This study
pCF11	pRW19e containing 218 bp *dvnA* signal peptide::*cbnA* PCR fusion product and 231 bp *cbiA* PCR product	This study
pCF12	pRW19e containing 200 bp *dvnA* signal peptide::*entB* PCR fusion product and 231 bp *cbiA* PCR product	This study
pCF13	pRW19e containing 218 bp *dvnA* signal peptide::*cbnA* PCR fusion product and 197 bp *eniB* PCR product	This study

* Ap^r, ampicillin resistant; Em^r, erythromycin resistant, Cm^r, chloramphenicol resistant.

multiple cloning site) and at a unique *Bsp*M1 restriction site located within the 3·1 kb fragment. The plasmid was blunt-ended and self-ligated. Plasmids pCF04 to pCF07 were prepared using the same deletion technique (Table 1, Fig. 1).

Preparation of the divergicin A signal peptide::carnobacteriocin A structural gene fusion. Creation of a divergicin A (*dvnA*) signal peptide::*entB* fusion construct was previously described (Franz *et al*., 1999) and this construct in plasmid pCMAP03 was transformed into *Ent. faecalis* ATCC 19433 to produce EntB by the general secretory pathway. To create the *dvnA* signal peptide::*cbnA* fusion construct the primers CF-01 (5′-TAT AAA GCT TCT GCT GAC CAA ATG TCA GAT GGT GT-3′) and CF-02 (5′-TAT ACT GCA GCA TGC GAG TTT TTT ATT TCA TAC AGC TA-3′) were used. Primer CF-01 contained a *Hin*dIII restriction site (underlined) which, together with the next 5 nucleotides (CTGCT), encodes the carboxy terminus of the DNA encoding the DvnA signal peptide (Worobo *et al*., 1995; McCormick *et al*., 1996). The nucleotides which follow CTGCT encode the amino terminus of mature CbnA and created an in-frame fusion of the 3′ end of the DvnA signal peptide DNA and the CbnA structural gene. Primer CF-02 contained adjacent *Pst*I and *Sph*I restriction sites (underlined) and it is complementary to the 3′ end of the CbnA structural gene. Using CF-01 and CF-02 as primers and pCF02 as template, DNA was amplified in 100 μl in 32 cycles (denaturation, 94 °C, 1 min; annealing, 54 °C, 1 min; extension, 72 °C, 1 min) using a temperature cycler (Omnigene, Intersciences). The PCR mixture contained 1·0 μM of each of the respective primers, 200 μM of dNTPs, 1 unit of *TaqPlus Precision* DNA polymerase and 1 × *TaqPlus Precision* buffer (Stratagene). The PCR product was cloned into the *Pst*I–*Hin*dIII sites of pUC118 for sequencing to confirm the fidelity of the reaction and for further cloning.

PCR amplification of the carnobacteriocin A and enterocin B immunity genes. *eniB* (the EntB immunity gene) was previously amplified by PCR and cloned into plasmid pMG36e to yield pCMAP05 (Franz *et al*., 1999) as shown in Fig. 2. *cbiA* (the CbnA immunity gene) was amplified by PCR using plasmid pCF02 as template. Primers CF-03 (5′-TAT ATC TAG AGA TCT AAT CAA AAT AAC TAG GA-3′) and CF-04 (5′-TAT AGG TAC CGT CTA CAG TCT GAA ACT AAA A-3′) were complementary to the 5′ and 3′ ends of this gene in pCF02, and contained *Xba*I and *Kpn*I restriction enzyme sites, respectively (underlined). This PCR reaction was done as described for the *dvnA* signal peptide::*cbnA* fusion above, except that an annealing temperature of 52 °C was used. The PCR product was cloned into pUC118 for sequencing to confirm the fidelity of the reaction. Plasmid pCF08 was created by cloning the *cbiA* PCR product into the *Xba*I–*Kpn*I sites of pMG36e under the control of the P32 promoter (Fig. 2).

Cloning of immunity genes behind the signal peptide::bacteriocin fusion genes and heterologous expression. The PCR amplicon that initiates the *dvnA* signal peptide::*cbnA* fusion was excised from pUC118 and cloned into the *Hin*dIII and *Kpn*I sites of pRW19e to displace the part of the *dvnA* operon that encodes mature DvnA and the DvnA immunity protein (McCormick *et al*., 1996; Franz *et al*., 1999) to create an in-frame fusion of CbnA with the DvnA signal peptide (pCF09). Plasmid pCF10 was created by digesting pCMAP03 with *Xba*I and *Kpn*I and inserting *eniB* (Franz *et al*., 1999) into these sites. *eniB* was originally cloned into the *Xba*I and *Hin*dIII sites of pUC118 but was excised by digesting with *Xba*I and *Kpn*I. Similarly, plasmid pCF11 was created by digesting pCF09 with *Xba*I and *Kpn*I and insertion of the *cbiA* gene. In plasmids pCF12 and pCF13 the immunity genes were exchanged (Table 1, Fig. 2). For heterologous or homologous expression of bacteriocins, plasmids pCF10 and pCF11 were

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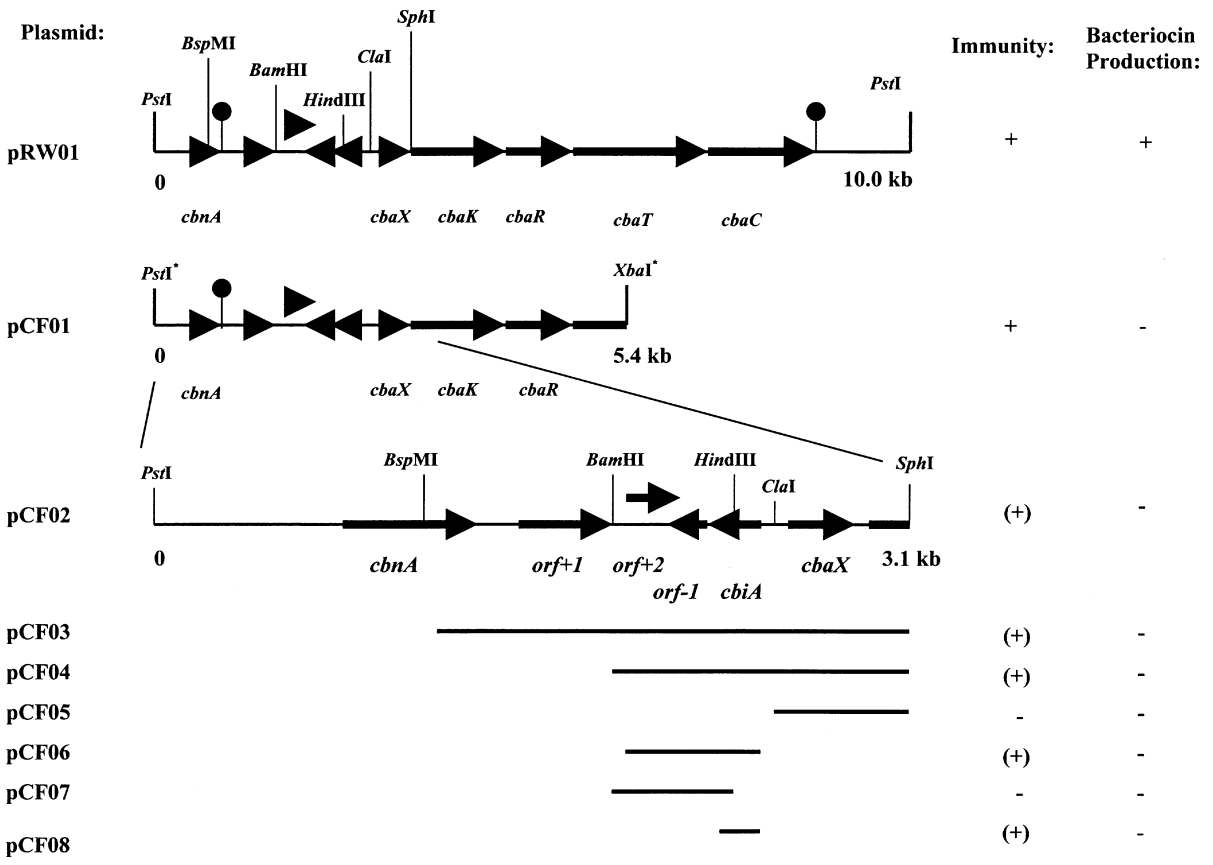

Fig. 1. Organization of genes involved in carnobacteriocin A production and immunity on plasmids pRW01 to pCF02, and schematic presentation of gene fragments in pCF03 to pCF08 to localize the carnobacteriocin A immunity gene. Bacteriocin activity and immunity of the constructs when transformed into *C. piscicola* LV17C is shown; (+) denotes partial immunity. Stem–loop vertical lines represent transcriptional terminators.

transformed into both *L. sakei* DSM 20017 and *C. piscicola* LV17C, and bacteriocin production was determined by deferred inhibition tests with *L. sakei* and *C. piscicola* strains containing pMG36e (Em^R) as indicators. For heterologous expression of immunity, *L. sakei* DSM 20017 containing pCMAP05 or pCF10, and *C. piscicola* LV17C containing pCF08 or pCF11, were used as indicators in deferred inhibition tests with *Ent. faecalis* ATCC 19433 containing pCMAP03 ($EntB^+$) and *C. piscicola* LV17A ($CbnA^+$) containing pMG36e as producers. To determine whether *eniB* and *cbiA* could confer cross-protection against CbnA and EntB, pCF12 was used for heterologous expression in *L. sakei* and pCF13 in *C. piscicola*. These clones were used as indicators in deferred inhibition assays with *Ent. faecalis* ATCC 19433 containing pCMAP03 and *C. piscicola* LV17A containing pMG36e as producers, respectively.

Induction factor synthesis and induction assays. The CbnA induction factor (CbaX) was synthesized at Anaspec Inc. (San Jose, CA, USA) and purified to >95% homogeneity by reverse-phase (C_{18}) HPLC using an acetonitrile/H_2O gradient. The molecular mass of the peptide was verified by electrospray mass spectrometry. To assay for biological activity a 1 mM stock solution of CbaX was prepared in sterile distilled water. To lose bacteriocin activity (Bac^-), a fully grown culture of *C. piscicola* LV17A was diluted 10^6-fold in 5 ml APT broth, incubated at 25 °C and allowed to grow until turbid. Cell-free supernatant was assayed for loss of bacteriocin activity against *C. piscicola* LV17C. This Bac^- culture was inoculated (1%) into APT broth containing CbaX at concentrations ranging from 10^{-5} to 10^{-14} mM and the subcultures were allowed to grow at 25 °C for 18 h and tested for bacteriocin production. Cell-free supernatant (1%) of *C. piscicola* LV17A containing CbnA (3200 AU ml^{-1}) was used in an induction test as a positive control.

RESULTS

Cloning and expression of the genes involved in carnobacteriocin A production

Transformation of *C. piscicola* LV17C with pRW01 resulted in full production of, and immunity to, CbnA. Plasmid pCF01, containing a 5·4 kb *Xba*I–*Pst*I fragment from pRW01, was transformed into *C. piscicola* LV17C and resulted in full immunity without CbnA production (Fig. 1). The complete nucleotide sequence of the 10 kb fragment was determined. It contains the previously sequenced 1·4 kb fragment encoding the CbnA structural gene and *orf+1* (Fig. 1), which was considered to

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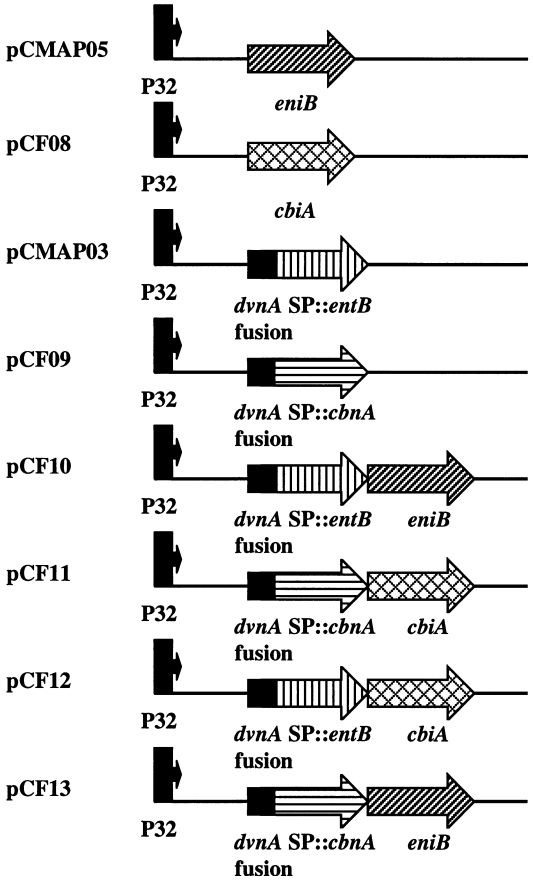

Fig. 2. Constructs used for testing production of carnobacteriocin A and enterocin B by the bacterial preprotein translocase and immunity to these respective bacteriocins. Black boxes denote the divergicin A signal peptide (*dvnA* SP), black boxes with an arrow indicate the P32 promoter.

be a candidate for the CbnA immunity gene (Worobo *et al*., 1994). Downstream of *orf+1* there are three small ORFs, one of which (*orf+2*) could encode a protein of 73 amino acids and partially overlaps *orf-1*. The other two ORFs, *orf-1* and *cbiA*, are on the opposite strand, with translational products of 38 amino acids (*orf-1*) and 56 amino acids (*cbiA*). The products of *orf+1*, *orf+2* and *orf-1* did not show homology to amino acid sequences reported in protein databases, while CbiA showed homology to the EntB immunity protein EniB (see below).

Downstream of these ORFs, the small ORF designated *cbaX* could encode a peptide of 41 amino acids. The deduced amino acid sequence of the N-terminus (residues 1–17) of CbaX showed homology to double-glycine-type leader peptides. Immediately downstream of *cbaX* are two further ORFs (*cbaK* and *cbaR*) which could encode proteins of 425 and 245 amino acids, respectively. The protein products translated from *cbaX*, *cbaK* and *cbaR* revealed significant homologies to established three-component regulatory systems of class II bacteriocins. The highest homology was observed for the regulatory system involved in carnobacteriocin B2 production. The translational product of *cbaX* showed 92% identity (96% similarity) to the induction factor associated with production of carnobacteriocin B2 in *C. piscicola* LV17B (Quadri *et al*., 1997). The translational products of *cbaK* and *cbaR* showed highest homology to the CbnB2 histidine protein kinase and response regulator (Table 2), as well as high homology to equivalent proteins of other regulated bacteriocins. An ORF designated *cbaT* that could encode a protein containing 716 amino acids is located immediately downstream of *cbaR*, and it is followed by an ORF designated *cbaC* which could encode a 455 amino acid protein. The translational products of *cbaT* and *cbaC* show high homology to ABC-transporter and accessory proteins involved in the production of other class II bacteriocins. CbaT and CbaC shared highest homology with the secretion proteins of CbnB2 (Quadri *et al*., 1997) and they also showed homology to transporter proteins involved in secretion of EntA (Table 2).

Each of these ORFs is preceded by probable RBS. The putative promoter upstream of *cbnA* was described by Worobo *et al*. (1994). A possible promoter sequence −35 (TTTAAT) and −10 (TACTTT) was found upstream of *cbaX*. Upstream of both *cbnA* and *cbaX* were conserved regulatory sequences consisting of right and left direct repeats. For *cbnA* the left (TTCAGG-ATA) and right (TTTAGGACA) direct repeat sequences are separated by 13 bases and the right repeat sequence was located 3 bases upstream of the presumptive −35 promoter sequence. For *cbaX* the left (TTCAAGACT) and right (TTCAGGATG) direct repeat sequences were also separated by 13 bases and the right repeat sequence was located three bases upstream of the presumptive −35 promoter sequence. Possible *rho*-independent terminators were identified after the *cbnA* and *cbaC* genes. No other ORFs could be identified in either strand of the 10·0 kb *Pst*I fragment.

Induction of CbnA production with chemically synthesized, mature CbaX

The translation product of the putative induction factor gene *cbaX* contained 41 amino acids; 17 of these were assumed to be the leader peptide, terminating with the typical Gly-Gly sequence, and the following 24 amino acids constituted the possible induction factor: SINSQ-IGKATSSISKCVFSFFKKC. The theoretical mass of this peptide was 2610·09 Da and the mass of the chemically synthesized CbaX determined by mass spectrometry was 2609·26 Da, possibly indicating a disulphide bridge between C^{16} and C^{24}. To determine whether the product of the *cbaX* gene is the induction factor for CbnA production, the chemically synthesized mature peptide was used to induce bacteriocin production in a Bac^- culture of *C. piscicola* LV17A. When the induction factor was added at a concentration of 10^{-11} M or higher, production of CbnA was induced, but not at concentrations of 10^{-12} M or lower. Similarly, supernatant from a Bac^+ culture of *C. piscicola* LV17A used as a positive control induced bacteriocin production.

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Table 2. Homology of ORFs from the carnobacteriocin A locus to those of the carnobacteriocin B2 and enterocin A and B loci

ORF	Homologous CbnB2 or EntA protein/peptide	Identity (%)	Similarity (%)	Function of protein/peptide
CbaX	CbaS	92	96	CbnB2 induction factor (Quadri *et al*., 1997)
	EntF	41	62	Enterocin A and B induction factor (Nilsen *et al*., 1998; O'Keeffe *et al*., 1999)
CbaR	CbnR	98	98	Carnobacteriocin B2 response regulator (Quadri *et al*., 1997)
	EntR	62	77	Enterocin A response regulator (O'Keeffe *et al*., 1999)
CbaK	CbnK	97	97	Carnobacteriocin B2 histidine kinase (Quadri *et al*., 1997)
	EntK	38	62	Enterocin A histidine kinase (O'Keeffe *et al*., 1999)
CbaT	CbnT	97	97	Carnobacteriocin B2 ABC transporter (Quadri *et al*., 1997)
	EntT	72	84	Enterocin A ABC transporter (O'Keeffe *et al*., 1999)
CbaC	CbnC	93	94	Carnobacteriocin B2 accessory protein (Quadri *et al*., 1997)
	EntD	46	64	Enterocin A accessory protein (O'Keeffe *et al*., 1999)

The Bac$^-$ culture, when inoculated into APT broth without induction factor, failed to produce bacteriocin and served as a negative control.

Cloning and expression of the gene conferring immunity to carnobacteriocin A

pCF01 lacked *cbaC* and the major part of *cbaT* and failed to produce bacteriocin, but it conferred full immunity when transformed into *C. piscicola* LV17C (Fig. 1). The 3·1 kb *Pst*I–*Sph*I fragment in pCF02 conferred partial immunity to *C. piscicola* LV17C when tested against *C. piscicola* LV17A, indicated by a smaller zone of inhibition compared with that obtained with the sensitive *C. piscicola* LV17C strain. The 3·1 kb fragment differed from the 5·4 kb fragment in that *cbnR* and the major part of *cbnK* were deleted (Fig. 1). Plasmid pCF03, which contains part of *cbnA* as well as *orf+1*, *orf+2*, *orf-1*, *cbiA* and *cbaX*, also conferred partial immunity to *C. piscicola* LV17C. Several other deletion derivatives were made (see Table 1 and Fig. 1) and *cbiA* was identified as the CbnA immunity gene. *cbiA* encodes a peptide of 56 amino acids which exhibits homology (30 % identity, 62 % similarity) with EniB, the 58 amino acid immunity peptide of EntB that is produced by *Ent. faecium* BFE 900 (Franz *et al*., 1999) (Fig. 3). *cbiA* was amplified by PCR and cloned into the pMG36e expression vector (pCF08). *cbiA* in pCF08 conferred the same partial immunity phenotype as that observed for *C. piscicola* LV17C containing pCF02, 03, 04 or 06. When *cbiA* was cloned together with either the *dvnA* signal peptide::*entB* or the *dvnA* signal peptide::*cbnA* gene fusion in *L. sakei* DSM 20017, full immunity was imparted to these heterologous hosts when tested against *C. piscicola* LV17A in the deferred inhibition tests (see below).

Expression of the divergicin A signal peptide::bacteriocin gene fusions

Plasmids pCF10 and 11 were constructed that encode the EntB or CbnA bacteriocins, respectively, fused to the signal peptide together with their cognate immunity protein. *C. piscicola* LV17C and *L. sakei* DSM 20017 containing plasmid pCF10 or 11 were tested for bacteriocin production against the indicators *L. sakei* DSM 20017 and *C. piscicola* LV17C. Zones of inhibition resulted when *L. sakei* DSM20017 was used as the indicator strain. When these constructs were tested against *C. piscicola* LV17C, activity resulted only from pCF11, indicating that *C. piscicola* LV17C is not sensitive to EntB. The EntB-producing, positive control *Ent. faecalis* ATCC 19433 was also not active against *C. piscicola* LV17C. The zone of inhibition produced by *L. sakei* DSM 20017 containing pCF11 was markedly smaller than that produced by *C. piscicola* LV17C containing this plasmid and noticeably smaller when tested against *L. sakei* DSM 20017 (8 vs 21 mm, respectively) or *C. piscicola* LV17C (17 vs 23 mm, respectively). The zone of inhibition produced by *C.*

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(a)  CbiA: MKTNLPKEMLILFSISIF----SNLILIFLTDTIIQKVLSSLSLIILLVVVCKEVKKNSN
            NL K  L  ::: IF     NL L:::::TI: K:L  :::::L::    : KK  N
     EniB:  MNLKKNNLE-YNLCIFLAVIINLGLFIFSETILSKILLLIAIVLLVIPNFMQKKKRKNS

(b)  CbnA:     -DQMSDGVNYGKGSSLSKGGAKCGLGIVGGLATIPSGPLGWLAGAAGVINSCMK
                          : ::LSKGGAKCG :I GGL  IP GPL:W AG A V : C
     EntB:     ENDHRMPNELNRPNNLSKGGAKCGAAIAGGLFGIPKGPLAWAAGLANVYSKCN-
```

Fig. 3. Alignment of (a) the carnobacteriocin A and enterocin B immunity proteins (CbiA and EniB, respectively) and (b) mature carnobacteriocin A and enterocin B (CbnA and EntB, respectively) by Lipman–Pearson alignment. Identical amino acids are shown; similar amino acids are indicated by a colon sign.

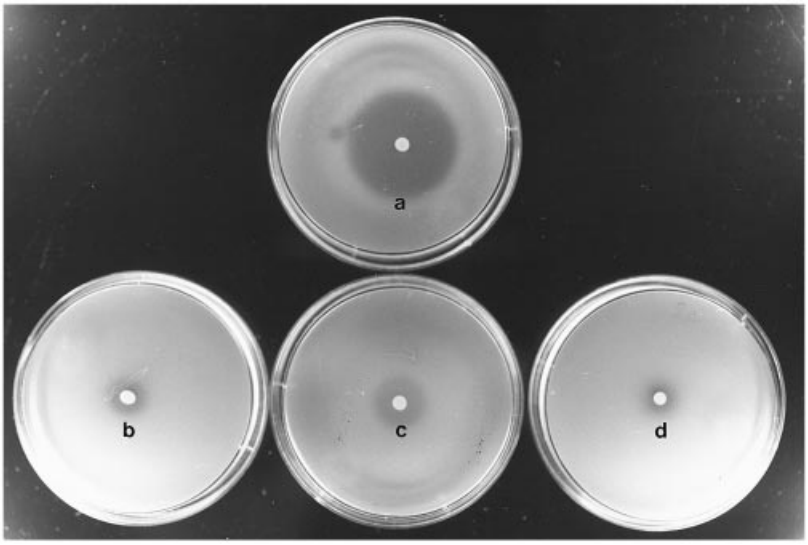

Fig. 4. Deferred inhibition tests with *C. piscicola* LV17A against *C. piscicola* LV17C (a) and *C. piscicola* LV17C containing pCF10 (b), pCF11 (c) and pCF13 (d).

piscicola LV17C containing pCF11 against both indicators was smaller than that produced by the positive control *C. piscicola* LV17A containing pCP49. The zone of inhibition produced by *C. piscicola* LV17C containing pCF10 was also markedly smaller than that of *L. sakei* DSM 20017 containing this construct when tested against the *L. sakei* DSM 20017 indicator strain (9 vs 17 mm, respectively), whereas the inhibition zone produced by the *L. sakei* DSM 20017 containing pCF10 was comparable to that produced by the EntB-producing positive control *Ent. faecalis* ATCC 19433 containing pCMAP03. *C. piscicola* LV17C and *L. sakei* DSM 20017 containing either plasmid pCF12 or 13 were also tested for bacteriocin production against the indicators *L. sakei* DSM 20017 and *C. piscicola* LV17C. These clones expressed the same bacteriocins; however, the immunity genes were interchanged (see below). Similar-sized zones of inhibition were observed as described above for these clones containing plasmids pCF10 or 11.

Expression of CbnA and EntB immunity genes and cross-immunity

C. piscicola LV17C containing plasmid pCF10, 11 or 13, and *L. sakei* DSM 20017 containing plasmid pCF10, 11 or 12, were tested as indicators for homologous or heterologous expression of immunity and cross-immunity with the EntB-producer *Ent. faecalis* ATCC 19433 containing pCMAP03, or the CbnA producer *C. piscicola* LV17A. *Ent. faecalis* ATCC 19433 containing pCMAP03 was active against *L. sakei* DSM 20017. Clones of *L. sakei* containing pCF10, 11 or 12 were fully immune to EntB. *C. piscicola* LV17C containing pCF10, 11 or 13 when tested as indicators in the deferred inhibition assay with *C. piscicola* LV17A as producer exhibited partial immunity, as shown by small zones of inhibition surrounding the producer (Fig. 4). The largest zone of inhibition obtained with these clones was for *C. piscicola* LV17C containing the CbnA immunity gene on pCF11. However, when plasmids pCF10, 11 or 12 were contained in *L. sakei* DSM 20017 they imparted full immunity to CbnA (data not shown).

DISCUSSION

CbnA is one of three bacteriocins produced by the wild-type *C. piscicola* LV17 (Worobo *et al.*, 1994). Initial characterization of CbnA defined it as a class II bacteriocin that is produced as a prebacteriocin con-

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taining an N-terminal extension that is cleaved following a Gly(-2)-Gly(-1) processing site. Subsequently, Holck *et al.* (1994) purified the identical bacteriocin from *C. piscicola* LV61 and designated it piscicolin 61. These reports provided information only on the primary structure of the bacteriocin and cloning of the bacteriocin structural gene. To achieve full production of CbnA, it was necessary to include the 8·6 kb of DNA downstream of the structural gene. It was originally postulated that this region contained the dedicated secretion machinery for CbnA; however, the results of this study show that, in addition to the two genes encoding the transport proteins, there are three genes associated with regulated production of bacteriocin. When the ORFs associated with CbnA production were analysed, highest homology was found with the CbnB2 secretion and regulation proteins, although the bacteriocins CbnA and CbnB2 show little homology. Based on their similarity to regulation and secretion proteins of other bacteriocin systems and, in particular, the striking similarity to those of CbnB2, the protein products of *cbaX*, *cbaK* and *cbaR* were assumed to be involved in CbnA regulation and those of *cbaT* and *cbaC* in the transport of CbnA. Evidence that the product of *cbaX* encodes an induction factor for CbnA regulation was obtained by chemical synthesis of mature CbaX devoid of the leader sequence and use of this peptide to induce CbnA production in a Bac^- culture of *C. piscicola* LV17A. CbaX is produced intracellularly as a prepeptide containing a 17 amino acid leader peptide of the double-glycine type, which is presumably removed by an ABC transporter during export from the cell. Bacteriocin production was induced at 10^{-11} M. Concentrations of extracellular signal molecules of Gram-positive bacteria are known to exert biological activity in the nanomolar range or below (Dunny & Leonard, 1997; Nilsen *et al.*, 1998).

Most class II bacteriocins have a dedicated immunity gene that immediately follows the structural gene in an operon-type arrangement, the product of which protects the cell from its own bacteriocin (Nes *et al.*, 1996). This was not the case for CbnA. It resembles the case of EntB production from *Ent. faecium* BFE 900 and T136, in which the immunity gene was not found in an operon together with the bacteriocin structural gene, but was located immediately downstream of, and in opposite orientation to, the bacteriocin structural gene (Franz *et al.*, 1999). For CbnA, *orf+1* was initially thought to encode the CbnA immunity protein (Worobo *et al.*, 1994); however, when a 700 bp *Stu*I–*Hin*dIII fragment containing this ORF was cloned and used in heterologous expression tests, immunity to CbnA was not observed (data not shown). In addition, absence of this ORF in pCF04 did not result in loss of the partial immunity phenotype. The gene conferring partial immunity to CbnA was identified by making sequential deletions of the 3·1 kb fragment in pCF02. When this gene (*cbiA*) was amplified by PCR and cloned into *C. piscicola* LV17C, it conferred the same level of partial immunity to this homologous host.

It is not clear why *cbiA* did not confer full immunity to *C. piscicola* LV17C, but a partial immunity phenotype has also been observed in other bacteriocin systems. Nisin immunity depends on the product of *nisI*, which is under common regulation with the *nisA* structural gene (Kuipers *et al.*, 1993). However, the *nisE*, *nisF* and *nisG* genes also are required for full immunity, and NisE and NisF show high homology to ABC transporter proteins (Siegers & Entian, 1995). The cyclic peptide bacteriocin AS-48 produced by *Ent. faecalis* S-48 requires the product of the *as-48D1* gene for immunity. In addition, the presence of the genes *as-48B*, *C1* and *D* is required for full immunity (Martínez-Bueno *et al.*, 1998). It was suggested that these genes encode subunits of an ABC transporter (As-48C1 and As-48D) as well as a protein (As-48B) involved with bacteriocin maturation (Martínez-Bueno *et al.*, 1998). The ABC transporter protein involved with transport of lacticin 481 in *Lactococcus lactis* strains is assembled from three subunits that are encoded by the genes *lctF*, *E* and *G*. The presence of these three genes imparted full immunity to lacticin 481 (Rincé *et al.*, 1997). Therefore, ABC transporter proteins also may have an immunity function in some bacteriocin systems. However, in the case of CbnA immunity, the association of transport proteins with immunity was considered unlikely. Only the N-terminal part of the putative ABC transporter of CbnA was present in plasmid pCF01. Yet, in homologous expression experiments, this plasmid conferred full immunity to CbnA.

The change from a full to a partial immunity phenotype occurred only in the absence of functional CbnA histidine kinase and response regulator genes, suggesting that production of CbnA immunity may be regulated. This is supported by similar observations that inactivation of regulatory genes decreases immunity. The presence of the response regulator gene *nisR* was required for nisin production and this gene was also shown to regulate nisin immunity (van der Meer *et al.*, 1993). For the sakacin A system it was shown that inactivation of the histidine kinase or response regulator genes resulted in the loss of immunity (Axelsson & Holck, 1995). For sakacin P, a frame-shift mutation in the histidine kinase gene also resulted in loss of immunity, while a deletion in the response regulator gene did not (Hühne *et al.*, 1996). These results indicate that the immunity phenotype can be subject to regulation and that the partial immunity phenotype observed for CbnA immunity is probably a result of basal-level expression of the immunity gene. However, heterologous expression of *cbiA* in *L. sakei* DSM 20017 resulted in full immunity of this host to CbnA. A difference in expression levels or efficiency of this gene in these hosts may explain this observation compared with only partial immunity when *cbiA* was cloned in *C. piscicola* LV17C. The mechanism of regulation of immunity requires further study to explain the partial immunity phenotype observed here.

CbiA consists of 56 amino acids, which is two amino acids less than EniB. CbiA and EniB are very similar in

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that they are hydrophobic peptides which contain charged amino acids at the amino and carboxy ends (Fig. 3). Using the PepTool protein structure prediction software both peptides were predicted to form an α-helix which may insert into the membrane. CbnA and EntB are *Listeria*-active bacteriocins, but they differ from other class II, especially class IIa bacteriocins, in that they do not contain a YGNGVXC consensus motif at the N-terminus of the mature bacteriocin peptide (Worobo *et al*., 1994; Casaus *et al*., 1997; Franz *et al*., 1999). The immunity proteins of these bacteriocins and the mature bacteriocins have sequence similarity (30 and 45 % identity, respectively) as shown in Fig. 3, and the immunity genes *cbiA* and *eniB* can be interchanged to cross-protect against EntB and CbnA. This is the first report of successful interchange of immunity genes in bacteriocin systems. The exact mechanism(s) by which immunity proteins function is not clear. It was suggested that immunity protein for CbnB2 blocked the pore formed by bacteriocins from the cytoplasmic side (Quadri *et al*., 1995). Other immunity proteins, e.g. LafI and LciM (van Belkum *et al*., 1991; Allison & Klaenhammer, 1996), were predicted to have transmembrane helices and therefore to be associated with the membrane. They may interact with the receptor for the bacteriocin and prevent binding of the bacteriocin to the membrane of the producer cell (Allison & Klaenhammer, 1996). EniB and CbiA also have an α-helical domain that may insert into the membrane and prevent bacteriocin binding. The homology observed between the mature CbnA and EntB and between their respective immunity proteins raises the question whether these bacteriocins should in future be considered as a novel class of bacteriocins.

ACKNOWLEDGEMENTS

These investigations were supported by the Natural Sciences and Engineering Research Council of Canada. C. M. A. P. F. gratefully acknowledges the financial assistance of a University of Alberta dissertation fellowship.

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Received 2 September 1999; revised 29 November 1999; accepted 9 December 1999.

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