Structure–function relationship of inducer peptide pheromones involved in bacteriocin production in *Carnobacterium maltaromaticum* and *Enterococcus faecium*

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The production of several bacteriocins in lactic acid bacteria is regulated by inducer peptide pheromones that specifically interact with their cognate bacterial receptor. These peptide pheromones are between 19 and 27 aa long and contain a conserved (V/I)-X-X-X-F sequence followed by positively charged residues in the C-terminal domain. CbaX and EntF are peptide pheromones that share similarity and are involved in the production of carnobacteriocin A in *Carnobacterium maltaromaticum* LV17A and enterocins A and B in *Enterococcus faecium* CTC492, respectively. CbaX, EntF and two hybrids, CbaX::EntF and EntF::CbaX, were tested for pheromone activity in LV17A and CTC492. EntF and EntF::CbaX only induced bacteriocin production in CTC492, whereas CbaX and CbaX::EntF induced carnobacteriocin A production in LV17A and, at high concentrations, also cross-induced enterocin production in CTC492. Various peptide fragments of CbaX and EntF were made for further structure–function analysis. The C-terminal fragments, but not the N-terminal fragments, were able to effect bacteriocin induction. The 10-mer EntF(16–25), derived from the C-terminal domain of EntF, showed pheromone activity in LV17A. In contrast, the C-terminal 9-mer of CbaX, CbaX(16–24), inhibited pheromone activity in both LV17A and CTC492. EntF(16–25) and CbaX(16–24) differ by two amino acids. Changing either one of these abolished pheromone activity as well as the ability to inhibit pheromone activity. These results indicate that the C-terminal domain of these peptide pheromones interacts relatively non-specifically with the receptor, and that induction is greatly facilitated by the N-terminal domain that recognizes specifically its cognate receptor.

INTRODUCTION

Many bacteria are able to communicate with each other in a cell-density-dependent manner, called quorum sensing, by the secretion of signalling molecules. These signalling molecules are detected by other bacteria when they reach a certain concentration and change the expression level of specific genes that enable the cells to adapt in a coordinated manner to their environment (Bassler & Losick, 2006; Keller & Surette, 2006; Reading & Sperandio, 2006). In Gram-positive bacteria, the signalling molecules are often inducer peptide pheromones (Kleerebezem & Quadri, 2001). The discovery that bacteriocin production in *Carnobacterium maltaromaticum* LV17 and *Lactobacillus plantarum* C11 was dependent on cell density indicated that quorum sensing plays a role in the production of class II bacteriocins in lactic acid bacteria (LAB) (Diep et al., 1995; Saucier et al., 1995). Class II bacteriocins are small, cationic, antibacterial peptides that do not contain unusual amino acids and permeabilize the membranes of sensitive, usually closely related bacteria (Klaenhammer, 1993; Nes et al., 1996; van Belkum & Stiles, 2000). It has been shown that the production of several, but not all, class II bacteriocins in LAB is regulated by peptide pheromones (van Belkum & Stiles, 2000). The gene encoding the peptide pheromone is often genetically linked to genes encoding the histidine protein kinase and the response regulator involved in bacteriocin expression (Kleerebezem & Quadri, 2001). The peptide pheromone is produced as a precursor with a double-glycine-leader peptide that is recognized and cleaved off by the bacteriocin ABC transporter during translocation out of the cell (Hävarstein et al., 1995; van Belkum et al., 1997). These peptide pheromones vary in length between 19 and 27 aa.
and are detected by the histidine protein kinase that is located in the cell membrane. The N-terminal domain of the histidine protein kinase contains six or seven membrane-spanning segments that act as the sensor of the peptide pheromone (Johnsberg et al., 2003). Interaction of the peptide pheromone with the histidine protein kinase activates the kinase, enabling it to phosphorylate the response regulator. The response regulator in turn activates the bacteriocin operon(s) (Kleerebezem & Quadri, 2001; Rohde & Quadri, 2006).

The interaction between receptor and peptide pheromone is highly specific and peptide pheromones only induce the cognate bacteriocin expression system of the receptor (Kleerebezem et al., 1997). In this study we investigated peptide pheromones from *Carnobacterium* and *Enterococcus* species that share some homology in their amino acid sequence. *C. maltaromaticum* LV17A, a derivative of strain LV17, produces the bacteriocin carnobacteriocin A (Ahn & Stiles, 1992; Worobo et al., 1994) and the production of this bacteriocin is regulated by the 24-mer peptide pheromone (Nilsen et al., 2000). Bacteriocins enterocin A and B are produced by a great variety of *Enterococcus faecium* strains (Franz et al., 2007). In several strains the expression of these two bacteriocins is regulated by the bacteriophage EmfF which consists of 25 aa. *E. faecium* CTC492 produces both enterocin A and B and both are induced by EmfF (Nilsen et al., 1998). Here we report that, at higher than normal concentrations of peptide pheromones, cross-induction between these two bacteriocin expression systems is possible and that the N-terminal domain of these peptides plays a role in recognition of its cognate receptor. Furthermore, we show that the C-terminal fragments of these peptide pheromones are able to cross-induce bacteriocin production or cross-interfere with pheromone activity, indicating that the C-terminal domain of these peptides interacts relatively non-specifically with the receptor molecule.

**METHODS**

**Induction peptide synthesis, purification and characterisation.**

Peptides were synthesized on pre-loaded resins [H-Cys(Trt)-2-Cl-Trt, Fmoc-Asn(Trt)-Wang or Fmoc-Ser(O-Bu)-Wang; Novabiochem] with resin loading between 0.54 and 0.75 mmol g⁻¹ on a 0.1–0.5 mmol scale. All peptides were synthesized manually using standard Fmoc solid-phase peptide synthesis (SPPS) protocols. Removal of Fmoc was completed using 20% piperidine/DMF for 5 min and was repeated until either a Kaiser test (Kaiser et al., 1970) was clearly positive or background absorbance was reached by UV detection at 301 nm. Side chain protecting groups for amino acids or derivatives were as follows: Asn(N-Trt), Glu(O-Bu), Gln(N-Trt), Lys(N-Boc), Ser(O-Bu) and Thr(O-Bu). Cysteine residues were introduced using Fmoc-Cys(Ttr)-OPfp esters. Pseudoproline (Mutter et al., 1995) (oxazolidine) dipeptides [Fmoc-Ala-Thr(p-MeMe)³-Pro-OH, Fmoc-Ser(O-Bu)-Ser(p-MeMe)³-Pro-OH, Fmoc-Phe-Ser(p-MeMe)³-Pro-OH; Novabiochem] were incorporated whenever possible, except when this would have resulted in contiguous couplings. Cleavage and deprotection of the peptide from the resin was accomplished by treatment with a freshly prepared solution of TFA/H₂O/trisopropylsilane (95:2.5:2.5, by vol.) mixture for 4 h with mechanical stirring. Filtration, followed by concentration in vacuo and trituration with cold diethyl ether afforded the crude peptides as white solids. Peptides were purified using a Delta-Pak C18 Prep-Pak Cartridge (Waters) installed on a Gilson HPLC (Gilson 322 pump, UV-Vis 152 detector; Mandel Scientific). Purification was achieved using acetonitrile and water containing 0.1% TFA at a flow rate of 10 ml min⁻¹ with UV detection at 220 nm. The acetonitrile gradient either was increased from 20 to 50% over 25 min followed by a ramp to 90% acetonitrile over 5 min, or was varied from 5 to 95% over 46 min. Fractions containing appropriate mass by MALDI-TOF MS were concentrated, lyophilized and repurified to homogeneity. Compounds were purified to single peak by HPLC and MALDI-TOF within 0.3 Da (M + H). All mass spectral analyses were performed on a Perspective Biosystems Voyager Elite MALDI-TOF mass spectrometer with delayed extraction in reflectron mode. The two-layer method (Dai et al., 1996, 1999) using 3.5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as the matrix was used for all MALDI-MS samples.

**Bacteriocin activity assay.** *C. maltaromaticum* strains LV17A and LV17C were grown in APT (All Purpose Tween) broth (Difco) at 25 °C. *E. faecium* CTC492 and *Lactobacillus sakei* DSM 20017 were grown in APT broth at 30 °C. Bacteriocin activity was determined using heat-treated cell-free supernatant of the producer organism in a spot-on-lawn test (Franz et al., 2000). The critical dilution method was used to measure bacteriocin titre (AU ml⁻¹) as described by Franz et al. (1996). *C. maltaromaticum* LV17C (Ahn & Stiles, 1992), a derivative of LV17A cured of its bacteriocin plasmid, was used as a sensitive indicator for carnobacteriocin A. *L. sakei* DSM 20017 was used as an indicator organism against enterocins A and B.

**Peptide pheromone activity.** To lose bacteriocin production, fully grown cultures of *C. maltaromaticum* LV17A and *E. faecium* CTC492 were diluted 10⁻⁶-fold in 5 ml APT and allowed to grow until turbid. Peptides to be tested for pheromone activity (see Table 1) were serial diluted in concentrations ranging from 10⁻⁵ to 10⁻¹³ M in 2.5 ml APT. A 1% inoculum of the bacteriocin non-producing culture LV17A or CTC492 was added to the broth containing the peptides and allowed to grow for 24 h. As a negative control, no peptides were added to bacteriocin non-producing cultures of LV17A and CTC492. The supernatants of these cultures were tested for bacteriocin activity by the spot-on-lawn assay using LV17C or DSM 20017 as indicators.

**Peptide fragments of CbaX and EntF to be tested for inhibition of pheromone activity of CbaX and EntF were added to 2.5 ml APT in concentrations ranging from 10⁻⁶ to 10⁻⁷ M. The peptide-containing APT broth was subsequently inoculated with 1% of a bacteriocin non-producing culture of LV17A or CTC492 and supplemented with 5 × 10⁻¹⁵ M CbaX or EntF, respectively. The bacteriocin titre of these cultures was measured after 24 h growth as described above using LV17C or DSM 20017 as indicators.

**Circular dichroism (CD) spectroscopy.** CD spectra were recorded using an Olis DSM 17 instrument. All measurements were obtained using a peptide concentration of 5 × 10⁻⁵ M in 10 mM potassium phosphate (pH 7.4) using various concentrations of trifluoroethanol (0–80%, v/v). Measurements were obtained at 23 °C using a quartz microcell cuvette (Hellma) with a path length of 0.2 mm. Samples were scanned five times at 20 nm min⁻¹ from 190 to 260 nm. The scans were averaged and corrected with respect to the baseline. The α-helical content of the peptide was estimated based on the value of the molar ellipticity (θ) at 222 nm using a modified equation from Morrow et al. (2000):

\[
\text{Percentage } \alpha\text{-helix} = \left(1 - \frac{\theta_{222} \text{nm} + 3000}{39000}\right) \times 100
\]
RESULTS

Pheromone activity of CbaX and EntF and their derivatives

The amino acid sequences of a number of inducer peptide pheromones responsible for the production of class II bacteriocins were compared (Fig. 1). Significant homology, especially in the C-terminal domain, can be seen between peptide pheromones produced by C. maltaromaticum and E. faecium. Furthermore, all peptides shown contain a conserved V/I-X-X-X-F sequence near the C-terminal end, followed by positively charged residues. Given the known specificity of peptide pheromones for their cognate receptors and the high homology between pheromones CbaX and EntF, we decided to investigate whether cross-induction could take place in C. maltaromaticum LV17A and E. faecium CTC492. Peptides CbaX and EntF were synthesized and used to induce bacteriocin production in both LV17A and CTC492 with peptide concentrations as high as $10^{-5}$ M. Both CbaX and EntF were able to induce their cognate bacteriocin production at $10^{-11}$ M in LV17A and CTC492, respectively (Table 1). EntF was unable to induce bacteriocin production in LV17A at a concentration of up to $10^{-5}$ M. However, CbaX induced bacteriocin production in CTC492 at $10^{-7}$ M (Table 1), indicating that at high concentrations this peptide pheromone is able to cross-induce bacteriocin production in E. faecium CTC492.

To determine the role of the N- and C-terminal domains of these peptides in pheromone activity, two hybrid peptides were made. CbaX: : EntF contains the first 10 N-terminal amino acids from CbaX and the C-terminal 15 amino acids from EntF, whereas EntF: : CbaX contains the first 10 N-terminal amino acids from EntF and the C-terminal 14 amino acids from CbaX (Table 1). Like EntF, EntF: : CbaX had no pheromone activity in LV17A. In contrast, bacteriocin production in LV17A could be induced by CbaX: : EntF at $10^{-3}$ M, indicating that the N-terminal domain is important for recognition by the cognate receptors.

Table 1. Amino acid sequences of various peptides and their ability to induce bacteriocin production in C. maltaromaticum LV17A and E. faecium CTC492

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
<th>Concentration of peptide (M) required for bacteriocin induction of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LV17A</td>
</tr>
<tr>
<td>CbaX</td>
<td>SINSQIKATSISISKCVFSSFKKC</td>
<td>$10^{-11}$</td>
</tr>
<tr>
<td>EntF</td>
<td>AGTKPQGKPASSLNECVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>CbaX: : EntF</td>
<td>SINSQIKATSISISKCVFSSFKKCN</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>EntF: : CbaX</td>
<td>AGTKPQGKPASSLNECVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>CbaX(7–24)</td>
<td>GKTSSISISKCVFSSFKKCN</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>CbaX(11–24)</td>
<td>SSISKCVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>CbaX(16–24)</td>
<td>CVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>CbaX(N–24 + N)</td>
<td>CVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>CbaX(1–11)</td>
<td>SINSQIKATS</td>
<td>–</td>
</tr>
<tr>
<td>EntF(7–25)</td>
<td>GKPASINLNECVFSSFKKCN</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>EntF(11–25)</td>
<td>SNLNECVFSSFKKCN</td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td>EntF(16–25)</td>
<td>CVFSSFKKCN</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>EntF(16–25)</td>
<td>CVFSSFKKCN</td>
<td>–</td>
</tr>
<tr>
<td>EntF(1–11)</td>
<td>AGTKPQGKPA</td>
<td>–</td>
</tr>
</tbody>
</table>

* EntF amino acid sequences are shown in bold type.

Fig. 1. Amino acid sequence alignment of inducer peptide pheromones involved in bacteriocin production in LAB. Conserved amino acids are shown in shaded blocks. * indicates the position of a conserved V/I residue. The peptide pheromones CbnS, CbaX, EntF, PisN, Orf4, IP-TX, SppIP and PIA are involved in the production of carnobacteriocin B2 in C. maltaromaticum LV17B (Kleerebezem et al., 2001) and enterocins A and B in E. faecium CTC492 (Nilsen et al., 1998), piscicolin 126 in C. maltaromaticum UAL26 (Gursky et al., 2006), sakacin A in L. sakei Lb706 (Axelsson & Holck, 1995), sakacin TX in L. sakei 5 (Vaughan et al., 2003), sakacin P in L. sakei LTH673 (Eijsink et al., 1996) and plantaricin A in L. plantarum C11 (Diep et al., 1995), respectively.
peptide fragments used in these inhibition studies was
and CTC492, respectively. The concentration of the
subsequently screened for pheromone activity in LV17A
CbaX and EntF (see Table 1). These peptides were
made based on the N-terminal 11 amino acids of CbaX and
EntF, respectively. As a result, all peptide fragments started
or ended with amino acid residues that are identical in
EntF, respectively. As a result, all peptide fragments started
and EntF(1–11) were
made based on the N-terminal 11 amino acids of CbaX and
EntF, respectively. A result, all peptide fragments started
or ended with amino acid residues that are identical in
CbaX and EntF (see Table 1). These peptides were
subsequently screened for pheromone activity in LV17A
and CTC492 at concentrations up to $10^{-5}$ M. CbaX(7–24)
and EntF(7–25) were able to induce their own cognate bacteriocin systems in LV17A and CTC492, respectively, at
a concentration of up to $10^{-6}$ M (Table 1). None of the
other CbaX fragments showed any pheromone activity in
LV17A or CTC492. However, the C-terminal 19-, 15- and
10-mers of EntF showed pheromone activity in LV17A
(Table 1). Neither of the N-terminal 11-mers of EntF and
CbaX was able to induce bacteriocin production (Table 1).
CbaX(16–24) and EntF(16–25) differ by only two amino
acids as EntF(16–25) has an additional asparagine residue
at its C terminus and a leucine residue instead of a
phenylalanine. Given the fact that EntF(16–25) but not
CbaX(16–24) induced bacteriocin production in LV17A,
two intermediate peptides, CbaX(16–24 + N) and EntF(16–
25 – N), were synthesized (Table 1). Neither of these two
peptides showed pheromone activity in LV17A and
CTC492, indicating that these single amino acid changes
in EntF(16–25) abolished pheromone activity.

All peptides described above were tested at a concentration
of $10^{-3}$ M for antimicrobial activity towards LV17C
and DSM 20017 using the spot-on-lawn technique. None of the
peptides caused inhibition of growth of the two LAB
strains (data not shown).

### C-terminal peptide fragment of CbaX inhibits pheromone activity

Various peptide pheromone fragments were tested for their
ability to inhibit pheromone activity by CbaX and EntF.
Both CbaX and EntF were used at a concentration of
$5 \times 10^{-11}$ M to induce bacteriocin production in LV17A
and CTC492, respectively. The concentration of the
peptide fragments used in these inhibition studies was
again up to $10^{-5}$ M. The two N-terminal fragments
CbaX(1–11) and EntF(1–11) as well as CbaX(11–24) and
EntF(11–25) were unable to interfere with bacteriocin
induction in LV17A and CTC492, respectively (data not
shown). However, the shortest C-terminal fragment of
CbaX was able to inhibit pheromone activity in both
strains. At $10^{-5}$ M, CbaX(16–24) reduced bacteriocin
production in LV17A and at up to $10^{-6}$ M the peptide
also reduced or completely inhibited bacteriocin production
in CTC492 (Table 2). In contrast, the shortest C-
terminal fragment of EntF, EntF(16–25), was unable to
inhibit pheromone activity in CTC492 (data not shown).
Neither CbaX(16–24 + N) nor EntF(16–25 N) affected
pheromone activity in LV17A and CTC492 (data not
shown), indicating that at the concentrations used only
CbaX(16–24) has the potential to antagonize pheromone
activity of CbaX and EntF.

### CD spectra analysis

The full-length peptides were dissolved at a concentration
of $5 \times 10^{-5}$ M in a variety of solvent mixtures of
trifluoroethanol (TFE) and $10^{-2}$ M potassium phosphate
buffer (pH 7.4), varying the TFE concentration from 0 to
80%. At concentrations of 0 and 20% TFE, the samples
did not assume any conformation consistent with z-helical
peptide structure (data not shown). At concentrations
of 40, 60 and 80% TFE, the CD spectra indicate that the
parent peptides CbaX and EntF display relatively constant
secondary structure ranging from 32–44% and 15–18% $\alpha$-
helical content, respectively (Table 3). It is interesting to
note that the CD spectra of the hybrid peptides respond
much more to TFE concentration. There is a much wider
range of $\alpha$-helical content in hybrids: 27–52% for the
CbaX::EntF hybrid and 9–28% for EntF::CbaX (Table 3).
In general, the secondary structures of CbaX and
CbaX::EntF show a higher $\alpha$-helical content in a
membrane-mimicking environment compared to EntF
and EntF::CbaX. These structural data appear to exhibit
a correlation to the activity of these peptides, as the CbaX
and CbaX::EntF peptides were both able to induce
bacteriocin production in LV17A and cross-induce at
similar levels bacteriocin production in CTC492. Similarly,
the CD spectra of EntF and EntF::CbaX correlate well with
their pheromone activity profile.

### Table 2. Inhibitory effect of CbaX(16–24) on pheromone activity of CbaX in C. maltaromaticum LV17A and EntF in E. faecium CTC492

<table>
<thead>
<tr>
<th>Concentration of CbaX(16–24) (M)</th>
<th>Bacteriocin production in LV17A induced by CbaX (AU ml$^{-1}$)</th>
<th>Bacteriocin production in CTC492 induced by EntF (AU ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0$</td>
<td>$6400$</td>
<td>$0$</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>$800$</td>
<td>$0$</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>$6400$</td>
<td>$200$</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>$6400$</td>
<td>$800$</td>
</tr>
</tbody>
</table>

[http://mic.sgmjournals.org](http://mic.sgmjournals.org)
In our study we were able to cross-induce the expression of enterocins in CTC492 with CbaX from C. maltaromaticum LV17A. To our knowledge this is the first example of cross-induction between different bacteriocin expression systems using non-hybrid histidine protein kinases. However, it probably cannot be described as cross-talk between species, as the amount of CbaX required to induce enterocin production in CTC492 is significantly higher (4 logs) than is required with EntF. When spent supernatant of LV17A is used, no bacteriocin induction in CTC492 is observed, indicating that the amount of CbaX produced by LV17A in the supernatant is not enough to activate enterocin production in CTC492 (data not shown).

Experiments with the N- and C-terminal fragments of CbaX and EntF showed that the C-terminal 18-mer of CbaX and 19-mer of EntF, but not the N-terminal 11-mers of CbaX and EntF were able to induce their cognate bacteriocin system in LV17A and CTC492. When shorter versions of the C-terminal 18- and 19-mer of CbaX and EntF were used, the peptides lost the ability to induce their cognate bacteriocin systems. These results seem to contradict studies with the peptide pheromone plantaricin A (Kristiansen et al., 2005). Plantaricin A is produced by L. plantarum C11 in three variants: as a 26 aa full-length peptide and as N-terminal truncated forms of 23 and 22 aa. Interestingly, all three variants of plantaricin A also have antimicrobial activity. Induction studies showed that at high concentrations, the N-terminal 5-mer of plantaricin A induced bacteriocin activity that was dependent on the chirality of the peptide, as only the L- but not the D-enantiomeric form had pheromone activity (Kristiansen et al., 2005). Furthermore, the L- and D-forms of the C-terminal 17-mer of plantaricin A were able to inhibit pheromone activity by plantaricin A, indicating that the inhibition of induction by these peptides was not dependent on chirality (Kristiansen et al., 2005). Unexpectedly, the C-terminal 19-, 15- and 10-mers of EntF cross-induced bacteriocin production in LV17A. The 10-mer had even more pheromone activity than the 19- or 15-mer peptides as the concentration of EntF(16–25) needed to induce carnobacteriocin A in LV17A was 10-fold lower than that of EntF(11–25) and EntF(7–25). This would indicate that the interaction of the C-terminal domain of these peptide pheromones is not necessarily very specific towards its own cognate receptor. This seems to be confirmed by the fact that the C-terminal 9-mer of CbaX, CbaX(16–24), is able to inhibit pheromone activity of CbaX as well as EntF in LV17A and CTC492, respectively. Apparently, the interaction of CbaX(16–24) with the receptor does not induce a conformational change that would activate the sensor, but the interaction is enough to compete with the pheromone activity of CbaX and EntF. The fact that these domains can interact with receptors from different bacteriocin systems might explain why chirality does not play a role in recognition of the C-terminal domain by the receptor as was observed for plantaricin A (Kristiansen et al., 2005). However, the results with CbaX(16–24 + N) and EntF(16–25 − N) show that a small change in the amino acid sequence can abolish interaction of the C-terminal domain with the receptor. NMR analysis of plantaricin A revealed that the C-terminal domain has an amphiphilic α-helical structure in a lipid environment, suggesting an electrostatic interaction with the membrane surface near the receptor (Kristiansen et al., 2005). The CD spectra of CbaX and CbaX::EntF show considerable α-helical content in the presence of structure-inducing solvents such as TFE, but this is less apparent for the other peptides synthesized in the current study. However, given the homology in the C-terminal domain of these peptide pheromones with plantaricin A, it is possible that a similar amphiphilic α-helical structure may be produced in the C-terminal domain of CbaX and EntF. The possibility that these peptide pheromones interact with the receptor via the membrane environment is in line with the suggestion that nisin, an amphiphilic lantibiotic bacteriocin produced by Lactococcus lactis, induces its own production by inserting itself in the membrane and activating the histidine protein kinase NisK (Abbas Hilmi et al., 2006).

The induction experiments in CTC492 with the full-length peptide pheromones and their hybrids revealed that the N-terminal domain plays a major role in the recognition of

**Table 3. α-Helical content of natural and hybrid peptide pheromones in varying concentrations of TFE in phosphate buffer (pH 7.4)**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>TFE: phosphate buffer (pH 7.4)</th>
<th>Molar ellipticity at 222 nm (%)</th>
<th>α-Helix (%) *</th>
</tr>
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<tbody>
<tr>
<td>CbaX</td>
<td>40</td>
<td>−9 320.36</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>−12 033.78</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>−14 014.60</td>
<td>44</td>
</tr>
<tr>
<td>CbaX::EntF</td>
<td>40</td>
<td>−17 421.24</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>−7 700.73</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>−12 476.15</td>
<td>40</td>
</tr>
<tr>
<td>EntF::CbaX</td>
<td>40</td>
<td>−3 444.39</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>−7 974.83</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>−643.17</td>
<td>9</td>
</tr>
<tr>
<td>EntF</td>
<td>40</td>
<td>−3 271.62</td>
<td>16</td>
</tr>
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<td></td>
<td>60</td>
<td>−2 965.30</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>−3 960.83</td>
<td>18</td>
</tr>
</tbody>
</table>

*Calculated as described in Methods.

**DISCUSSION**

Inducer peptide pheromones play an essential role in the production of a number of bacteriocins in LAB. These peptide pheromones specifically induce their cognate bacteriocin expression system (Kleerebezem et al., 1997). In our study we were able to cross-induce the expression of enterocins in *E. faecium* CTC492 with CbaX from *C. maltaromaticum* LV17A. To our knowledge this is the first example of cross-induction between different bacteriocin expression systems using non-hybrid histidine protein kinases. However, it probably cannot be described as cross-talk between species, as the amount of CbaX required to induce enterocin production in CTC492 is significantly higher (4 logs) than is required with EntF. When spent supernatant of *C. maltaromaticum* LV17A is used, no bacteriocin induction in CTC492 is observed, indicating that the amount of CbaX produced by LV17A in the supernatant is not enough to activate enterocin production in CTC492 (data not shown).

Experiments with the N- and C-terminal fragments of CbaX and EntF showed that the C-terminal 18-mer of CbaX and 19-mer of EntF, but not the N-terminal 11-mers of CbaX and EntF were able to induce their cognate bacteriocin system in LV17A and CTC492. When shorter versions of the C-terminal 18- and 19-mer of CbaX and EntF were used, the peptides lost the ability to induce their cognate bacteriocin systems. These results seem to contradict studies with the peptide pheromone plantaricin A (Kristiansen et al., 2005). Plantaricin A is produced by *L. plantarum* C11 in three variants: as a 26 aa full-length peptide and as N-terminal truncated forms of 23 and 22 aa. Interestingly, all three variants of plantaricin A also have antimicrobial activity. Induction studies showed that at high concentrations, the N-terminal 5-mer of plantaricin A induced bacteriocin activity that was dependent on the chirality of the peptide, as only the L- but not the D-enantiomeric form had pheromone activity (Kristiansen et al., 2005). Furthermore, the L- and D-forms of the C-terminal 17-mer of plantaricin A were able to inhibit pheromone activity by plantaricin A, indicating that the inhibition of induction by these peptides was not dependent on chirality (Kristiansen et al., 2005). Unexpectedly, the C-terminal 19-, 15- and 10-mers of EntF cross-induced bacteriocin production in LV17A. The 10-mer had even more pheromone activity than the 19- or 15-mer peptides as the concentration of EntF(16–25) needed to induce carnobacteriocin A in LV17A was 10-fold lower than that of EntF(11–25) and EntF(7–25). This would indicate that the interaction of the C-terminal domain of these peptide pheromones is not necessarily very specific towards its own cognate receptor. This seems to be confirmed by the fact that the C-terminal 9-mer of CbaX, CbaX(16–24), is able to inhibit pheromone activity of CbaX as well as EntF in LV17A and CTC492, respectively. Apparently, the interaction of CbaX(16–24) with the receptor does not induce a conformational change that would activate the sensor, but the interaction is enough to compete with the pheromone activity of CbaX and EntF. The fact that these domains can interact with receptors from different bacteriocin systems might explain why chirality does not play a role in recognition of the C-terminal domain by the receptor as was observed for plantaricin A (Kristiansen et al., 2005). However, the results with CbaX(16–24 + N) and EntF(16–25 − N) show that a small change in the amino acid sequence can abolish interaction of the C-terminal domain with the receptor. NMR analysis of plantaricin A revealed that the C-terminal domain has an amphiphilic α-helical structure in a lipid environment, suggesting an electrostatic interaction with the membrane surface near the receptor (Kristiansen et al., 2005). The CD spectra of CbaX and CbaX::EntF show considerable α-helical content in the presence of structure-inducing solvents such as TFE, but this is less apparent for the other peptides synthesized in the current study. However, given the homology in the C-terminal domain of these peptide pheromones with plantaricin A, it is possible that a similar amphiphilic α-helical structure may be produced in the C-terminal domain of CbaX and EntF. The possibility that these peptide pheromones interact with the receptor via the membrane environment is in line with the suggestion that nisin, an amphiphilic lantibiotic bacteriocin produced by *Lactococcus lactis*, induces its own production by inserting itself in the membrane and activating the histidine protein kinase NisK (Abbas Hilmi et al., 2006).

The induction experiments in CTC492 with the full-length peptide pheromones and their hybrids revealed that the N-terminal domain plays a major role in the recognition of
the peptide pheromone by its cognate receptor. The results with CbaX and CbaX::EntF showed that replacing the EntF N-terminal domain by the CbaX N-terminal domain greatly reduced pheromone activity in CTC492. The ability to induce bacteriocin production in LV17A was completely abolished when the CbaX N-terminal domain was replaced by the EntF N-terminal domain in EntF::CbaX and EntF. These results, and the finding that the chirality of the N-terminal domain of plantaricin A is important for pheromone activity (Kristiansen et al., 2005), indicate that the specific interaction of the N terminus with the receptor explains why these peptide pheromones only induce their cognate bacteriocin system. Taken together, peptide pheromones seem to use their amphiphilic C-terminal domain to bind to the histidine protein kinase, possibly at membrane level, in a relative non-specific fashion. The weak interaction is greatly improved by the N-terminal domain of the peptide pheromone that is highly specific for the cognate receptor. This interaction will enable the peptide pheromone to activate bacteriocin production at less than nanomolar level.

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