Role of Acetate in Production of an Autoinducible Class IIa Bacteriocin in *Carnobacterium piscicola* A9b

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*Carnobacterium piscicola* strain A9b isolated from cold smoked salmon inhibits growth of the food-borne pathogen *Listeria monocytogenes* partly due to the production of a proteinaceous compound (L. Nilsson, L. Gram, and H. H. Huss. J. Food Prot. 62:336-342, 1999). The purpose of the present study was to purify the compound and describe factors affecting its production, with particular consideration of food-relevant factors. Amino acid sequencing showed that the compound is a class IIa bacteriocin with an N-terminal amino acid sequence identical to that of carnobacteriocin B2. The production of the bacteriocin was autoinducible, and the threshold level for induction was $9.6 \times 10^{-10}$ M. We also report, for the first time, that acetate acts as an induction factor, with a threshold concentration of 0.3 to 12 mM. Acetate could not act as an inducer during the late exponential phase of *C. piscicola* A9b. The induction of bacteriocin production showed a dose-dependent relationship at acetate concentrations of up to 10 to 20 mM (depending on the growth medium) and at a concentration of $1.9 \times 10^{-8}$ M for the bacteriocin itself; a saturation level of bacteriocin specific activity was reached at these concentrations of induction factors. The combined use of both inducers did not enhance the saturation level of bacteriocin production compared to that seen with the use of each inducer alone. Increasing NaCl and glucose concentrations negatively influenced the efficiency of acetate as an induction factor. Based on the results, carnobacteriocin B2 was used as an induction factor to manipulate the production of bacteriocin in cold smoked salmon juice and thus improve the ability to inhibit *L. monocytogenes*.

The ubiquitous nature of *Listeria monocytogenes*, its ability to survive and grow in a wide range of food products, and the severity of the disease it causes (20 to 30% fatality) have heightened the awareness of this pathogen as a public health problem over the past decade (13). Research on the control of this pathogen has expanded significantly. In addition to traditional preservation techniques, several new preservation strategies have been developed to inhibit its growth in food products, thus preventing future outbreaks. The use of bacteriocinogenic lactic acid bacteria (LAB) and/or their isolated bacteriocins may offer a promising solution (8, 10, 27).

A number of studies have been conducted on class IIa bacteriocins, which are highly active against *Listeria* species. Class IIa bacteriocins, also known as pediocin-like bacteriocins, are small, heat-stable, non-lanthionine-containing peptides that contain at least two cysteines with disulfide bridges. The primary structure of class IIa bacteriocins is characterized by a conserved N-terminal hydrophilic domain which has the consensus sequence YGNGV(Xaa)C(Xaa)4V-(Xaa)4A (where Xaa is any amino acid) and a highly variable C-terminal hydrophobic region (12). Class IIa bacteriocins do not undergo posttranslational modification, except for cleavage of the leader peptides across the cytoplasmic membrane and the formation of disulfide bridges. These leader peptides contain a conserved double-glycine motif in the C terminus, and this motif is believed to serve as a signal peptide for the processing and secretion of bacteriocins by an independent ATB-binding cassette transporter (24, 44).

The production of most class IIa bacteriocins is regulated by a three-component system which includes a histidine protein kinase, a response regulator, and an induction factor. Some class IIa bacteriocins are autoregulated by a two-component signal transduction system (32), which is a well-known phenomenon in lantibiotics (37). A threshold concentration of the bacteriocin, which functions as a signal molecule accumulating during growth, triggers the transcription of the genes coding for bacteriocin production, suggesting a self-inducing cell density (quorum-sensing)-regulated system (32, 37).

Bacteriocin production is influenced by several environmental factors, such as pH (1, 25), temperature (9), and NaCl (20, 43) and ethanol (23) concentrations. These environmental factors may influence growth negatively and thereby the secretion of the induction factor (9). Further, it has been suggested that some environmental factors reduce the binding of the induction factor to its receptor (25). Understanding the influence of food-related environmental factors on the induction of bacteriocins is essential for the effective commercial application of bacteriocin-producing LAB in the preservation of foods.

Several species within the genus *Carnobacterium*, which forms a distinct phylogenetic clade within the LAB, produce class IIa bacteriocins (carnobacteriocins BM1 and B2 and piscicolin 126). Carnobacteria are associated with chilled vacuum-packed meat, poultry, and fish products (2, 7, 16). In a previous study, *Carnobacterium piscicola* strain A9b isolated from cold smoked salmon was used for the biopreservation of vacuum-packed cold smoked salmon (27). *C. piscicola* A9 was able to inhibit *L. monocytogenes* both in model experiments with cold smoked salmon juice and in vacuum-packed cold...
The competitive ability of *C. piscicola* strain A9b was attributed to rapid growth in the food system and the production of antilisterial substances. Further development of this strain for the biopreservation of food products requires an understanding of the mechanisms of action of the antilisterial activity and of the influence of food-related parameters on the inhibitory action. Here we report on the isolation and characterization of a class IIa bacteriocin produced by *C. piscicola* A9b. We show that acetate and the bacteriocin itself function as dose-dependent inducers of bacteriocin production both in laboratory media and in cold smoked salmon juice.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and media.** The bacteriocin producer, *C. piscicola* strain A9b was isolated from vacuum-packed cold smoked salmon stored at 5°C (29). A bacteriocin-negative (Bac−) mutant of *C. piscicola* A9b was created by exposure to 5, 10, 15, and 20 μg of acriflavin/ml (26). *L. monocytogenes* strain O57 (3) and *C. mesenteroides* strain O57 (3) and *Pediococcus acidilactici* TA33a (30), *Lactobacillus sake* MI401 (18) (reclassified from *Lactobacillus buvaricus*) and *Enterococcus faecium* TS86 (4).

All strains were propagated twice in broth before use. *C. piscicola* and *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Oxoid Ltd., Hampshire, England; product code CM225) at 25°C for 18 h. *L. acidilactici* and *L. sake* were grown in deMan-Rogosa-Sharpe (MRS) broth (CM359, Oxoid) at 30°C for 18 h before use. *E. faecium* was grown in BHI broth at 30°C. Working cultures were maintained at 5°C on BHI agar (BHI broth containing 1.2% agar; Bié & Berntsen A/S, Høje Taastrup, Denmark; code CB180030) or MRS agar (Oxoid; code CM361), and frozen stock cultures were stored at −80°C.

For bacteriocin production studies, a 1.0 or 0.001% stationary-phase culture of *C. piscicola* A9b (incubation at 25°C for 18 h) was inoculated into MRS7–GCA, MRS7–CA, or MRS7–AG broth (Oxoid; see below) or all-purpose Tween (APT) broth (Difco; 0655-17-9). MRS7–GCA broth was prepared as described previously (15) by assembling the individual components of MRS broth in accordance with the manufacturer’s formula but with the exclusion of glucose, triammonium citrate, and sodium acetate from the medium. The components were dissolved in distilled water and the pH was adjusted to 7.0 (MRS7 broth) before autoclaving. Tween 80, dipotassium hydrogen phosphate, magnesium sulphate, and magnesium chloride were added as sterile filtered solutions after autoclaving. In MRS7–CA broth and MRS7–AG broth, glucose and citrate were added, respectively.

Cold smoked salmon juice was made as described by Nilsson et al. (27). Salt analysis was performed as described previously (1a), and NaCl was added to a final concentration of 4.0% (wt/vol). The juice was heat treated for 30 min at 100°C and stored in the dark at 5°C for a maximum of 3 days before use.

**Assays for bacteriocin and induction activity.** The bacteriocin concentration was determined in an agar diffusion assay (28) by using 10^6 CFU of the indicator organism/ml in 48°C BHI agar (BHI broth containing 0.8% agar) supplemented with 0.1% Tween 80 (Merck; code 22817) and poured into 14-cm petri dishes (Sterilin, Bibby, Sterilin Ltd., Stone, Staffs, United Kingdom). Supernatant fluid from the producer strain, *C. piscicola* A9b, was adjusted to pH 6.5 with 2 N NaOH and filter sterilized (0.2-μm-pore-size nylon membrane filter; Sartorius; 16534K). Samples (50 μl) were pipetted into 7-mm wells cut into the agar. The plates were preincubated at 5°C for 24 h followed by 18 h at 25°C. Twofold dilution series of supernatant fluid were analyzed (28); the reciprocal of the highest dilution causing a zone of inhibition of the indicator organism was expressed as the number of bacteriocin units (BU) per milliliter and 1 IU was arbitrarily defined as the minimum concentration of induction factor resulting in detectable bacteriocin production.

**Stability of bacteriocin and an extracellular induction factor produced by *C. piscicola* A9b.** Bacteriocin produced by *C. piscicola* A9b was purified from BHI broth, although optimal bacteriocin production was observed in MRS7 broth (15). However, it was observed that MRS7 broth interferes with the purity of bacteriocin due to the presence of the detergent Tween (5). *C. piscicola* A9b was grown in BHI broth to the stationary phase (24 h at 25°C), corresponding to an optical density (OD) of 1.3 to 1.4. Ammonium sulfate (Merck; code 101217) was added to a cell-free supernatant (40% [wt/vol]), and the mixture was stirred for 20 h at 2°C. The bacteriocin precipitate was harvested (10,000 × g for 30 min), resuspended in 50 mM sodium-acetate buffer (pH 4.5), and desalted by dialysis against the same phosphate buffer (cutoff, 1,000; Spectrum Laboratories, Inc., Rancho Dominguez, Calif.). The bacteriocin was purified by cation-exchange chromatography with a HiTrap SP column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM acetate buffer (pH 4.5). The bacteriocin was eluted as a single band with a flow rate of 5.0 ml/min with a linearly increasing gradient (starting with 50 mM sodium-acetate buffer [pH 4.5] and ending with 50 mM sodium-acetate buffer [pH 4.5] containing 1 M NaCl). The active fraction was applied to a hydrophobic interaction column (HiLoad 16/10 Phenyl-Sepharose; Amersham Pharmacia Biotech) or a SMART system (Amersham).

**Production, purification, and amino acid sequencing of bacteriocin and an extracellular induction factor produced by *C. piscicola* A9b.** Bacteriocin produced by *C. piscicola* A9b was purified from BHI broth, although optimal bacteriocin production was observed in MRS7 broth (15). However, it was observed that MRS7 broth interferes with the purity of bacteriocin due to the presence of the detergent Tween (5). *C. piscicola* A9b was grown in BHI broth to the stationary phase (24 h at 25°C), corresponding to an optical density (OD) of 1.3 to 1.4. Ammonium sulfate (Merck; code 101217) was added to a cell-free supernatant (40% [wt/vol]), and the mixture was stirred for 20 h at 2°C. The bacteriocin precipitate was harvested (10,000 × g for 30 min), resuspended in 50 mM sodium-acetate buffer (pH 4.5), and desalted by dialysis against the same phosphate buffer (cutoff, 1,000; Spectrum Laboratories, Inc., Rancho Dominguez, Calif.). The bacteriocin was purified by cation-exchange chromatography with a HiTrap SP column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM acetate buffer (pH 4.5). The bacteriocin was eluted as a single band with a flow rate of 5.0 ml/min with a linearly increasing gradient (starting with 50 mM sodium-acetate buffer [pH 4.5] and ending with 50 mM sodium-acetate buffer [pH 4.5] containing 1 M NaCl). The active fraction was applied to a hydrophobic interaction column (HiLoad 16/10 Phenyl-Sepharose; Amersham) and eluted with a linear gradient of 8% ammonium sulfate. The active fraction was used for final purification by reverse-phase high-pressure liquid chromatography (HPLC) with a C4-C8 Nucleosil column (250 by 4.6 mm) and a linear gradient from 100% 0.1% trifluoroacetic acid to 90% acetonitrile in 0.1% trifluoroacetic acid. Chromatographic purification was performed at room temperature by fast protein liquid chromatography (Bio-Rad, Copenhagen, Denmark) with a SMART system (Amersham).

Protein content, estimated by measuring the A280 nm bacteriocin activity (*L. monocytogenes* O57 was used as the target strain), and induction activity were determined at each step of the purification process.

**Induction studies.** All induction experiments described below were done at 25°C. Cell density was determined by measuring of the OD at 600 nm (OD600) (Novaspec II; Amersham). Bacteriocin activity was determined when cultures had reached the stationary phase of growth (22 to 48 h).

The influence of carbon sources (acetate, citrate, and glucose), extracellular compounds produced by *C. piscicola* A9b, and inoculum size on both cell density and the production of bacteriocin by *C. piscicola* A9b was investigated. Acetate (Sigma; S7670), citrate (Sigma; A1332), and glucose (Sigma; G7528) were added to the basal medium (MRS7–GCA broth) at final concentrations of 36.74, 8.22, and 111.01 mM, respectively, corresponding to the concentrations in manufactured MRS broth. In some combinations, sterile filtered supernatant from the...
bacteriocin-producing strain (Bac\(^–\)) or the non-bacteriocin-producing mutant (Bac\(^+\)) of \textit{C. piscicola} A9b was added at a concentration of 1.0%. A stationary-phase culture of \textit{C. piscicola} A9b (incubation at 25°C for 18 h) was used as an inoculum for each medium at a 0.001% (vol/vol) or a 1.0% (vol/vol) final concentration.

The induction capacity of acetate and an extracellular proteinaceous factor produced by \textit{C. piscicola} A9b was investigated with MR57—CA, MR57—CA broths without the addition of citrate, or APT broth. Acetate was added to the broth at increasing concentrations ranging from 0 to 90 mM, and the extracellular induction factor, obtained from a sterile, cell-free culture supernatant of \textit{C. piscicola} A9b (see the description of the induction assay), was added to the broth at increasing concentrations ranging from 0 to 4.06 kIU ml\(^–1\). A stationary-phase culture of \textit{C. piscicola} A9b was used as an inoculum at 0.001% (vol/vol). Induction by the extracellular proteinaceous factor was examined in duplicate. Induction by acetate was examined in a single experiment; however, representative points were examined in an independent experiment, and results from the first experiment were confirmed.

We investigated if the induction capacity of acetate was influenced by the time at which acetate was added to the growth medium of \textit{C. piscicola} A9b. Acetate corresponding to a final concentration of 36.74 mM was added to MR57—CA broth every second hour (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h) after inoculation with \textit{C. piscicola} A9b—(0.001% inoculum from a stationary-phase culture).

The effects of glucose and NaCl on the induction capacity of acetate and the proteinaceous induction factor produced by \textit{C. piscicola} A9b were investigated as follows. (i) Acetate and glucose were added to MR57—CA broth at different concentrations (from 0 to 36.74 mM and from 0 to 111.11 mM, respectively). (ii) Acetate and the extracellular induction factor were added to MR57—CA broth containing increasing concentrations of NaCl (from 0 to 7.0%). (iii) Acetate and the extracellular induction factor were added to salmon juice at different concentrations (from 0 to 36.74 mM and from 0 to 111.11 mM, respectively). We examined if acetate could induce bacteriocin production in other LAB. L. gelidum UAL187-22, L. mesenteroides TA33a, P.acidilactici PA-2, L. sake MI401, and E. faecium TS86.

**Influence of metabolic conversion of glucose and production of acetate on \textit{C. piscicola} A9b bacteriocin production.** The production of acetate and the consumption of glucose by \textit{C. piscicola} A9b in MR57—GCA broth, MR57—CA broth, or salmon juice were determined with enzymatic kits (R-Biopharm; Boehringer Mannheim GmbH, Darmstadt, Germany).

**Inhibition of \textit{L. monocytogenes} OS7 in salmon juice by \textit{C. piscicola} A9b in the presence of induction factors.** \textit{C. piscicola} A9b was preincubated at 5°C for 5 days in BHI broth (1% precurate) supplemented with 4.0% (wt/vol) NaCl (27). \textit{L. monocytogenes} OS7 was grown at 15°C for 24 h in BHI broth (1% inoculum from a precurate) supplemented with 3.0% (wt/vol) NaCl (27) and diluted in 0.1% peptone water to appropriate cell numbers. Cold-smoked salmon juice with 4.0% (wt/vol) NaCl was inoculated with \textit{C. piscicola} A9b corresponding to inoculum levels of 1.0 and 0.001%. \textit{L. monocytogenes} OS7 was added at a final concentration of 10\(^5\) CFU/ml. A sterile filtered supernatant from bacteriocin-producing strain A9b was added (1.0%) to some of the cultures. The flasks were incubated for 27 days at 5°C in the dark. Samples were removed once or twice a week for the determination of colony counts and bacteriocin production.

\textit{L. monocytogenes} was enumerated by direct plating of 0.1 ml of appropriate dilutions onto listeria selective agar base (Oxoid; code CM856) to which listeria selective supplement (Oxoid; code SR140) had been added. The plates were incubated at 25°C for 48 h. Cell numbers of \textit{C. piscicola} were estimated by direct plating on nitrate-polyoxymyxin agar made from APT agar (Difco; 0654-17) (pH 6.7). The plates were incubated at 25°C for 48 h.

**RESULTS**

**Stability of the bacteriocin from \textit{C. piscicola} A9b.** The inhibitory compound produced by \textit{C. piscicola} A9b was inactivated by the proteolytic enzymes proteases I, IV, and XIV, proteinase K, and trypsin IX, suggesting a proteinaceous nature of the compound (bacteriocin). No significant decrease in activity was found after treatment with pepsin, lipase VII, phospholipase C, or α-amylase. The bacteriocin activity was stable at \(–20\), 0, and 5°C for 1 month and during heat treatment at 100°C for 10 min. However, the bacteriocin lost 50% of the initial activity (10,240 BU ml\(^–1\)) after 1 month of storage at 25°C. The bacteriocin activity was stable at pHs of between 2 and 5, but activity was lost at increasing pHs of 6 to 11.

**Bacteriocin production, purification, and amino acid sequence.** The bacteriocin produced by \textit{C. piscicola} A9b was purified by ammonium sulfate precipitation, cation-exchange chromatography, hydrophobic interaction chromatography (HIC), and reverse-phase chromatography. The HIC step resulted in a single A\(_{280}\) peak, and SDS-PAGE analysis showed an electromorphoretically pure peptide. However, since a desalting procedure (ultrafiltration) resulted in only 10% recovery of the active material (data not shown), a final separation by reverse-phase HPLC was performed. Reverse-phase HPLC yielded one absorbance peak coincident with the activity peak (data not shown), and the entire purification process resulted in an approximately 1.4 \(\times\) 10\(^4\)-fold increase in specific activity (data not shown). SDS-PAGE analysis of the peptide obtained from cation-exchange chromatography showed a molecular mass of approximately 4.5 kDa and inhibitory activity against \textit{L. monocytogenes} OS7 (data not shown).

**Results**

<table>
<thead>
<tr>
<th>Component(s) added to MR57—GCA broth</th>
<th>Bacteriocin sp act (10(^5) BU/OD(_{590}) unit) under the indicated conditions(^a)</th>
<th>Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td>1.0% Inoculum</td>
</tr>
<tr>
<td>1% Bac(^–) strain(^a)</td>
<td>6.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0–0.2</td>
<td>0.2–0.5</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Acetate</td>
<td>11.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Glucose + citrate</td>
<td>0–0.2</td>
<td>0–0.2</td>
</tr>
<tr>
<td>Acetate + glucose</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Acetate + citrate</td>
<td>11.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Acetate + glucose + citrate</td>
<td>8.9</td>
<td>9.4</td>
</tr>
</tbody>
</table>

\(^{a}\) Values are means for duplicate samples from independent cultures.

\(^{b}\) Bacteriocin production was detected after 24 h of incubation.

\(^{c}\) The filter-sterilized supernatant of the Bac\(^–\) strain contained 13.14 µmol of acetate per ml and 0 BU ml\(^–1\).

\(^{d}\) The filter-sterilized supernatant of the Bac\(^–\) strain contained 13.08 µmol of acetate per ml and 80 BU ml\(^–1\).

Amino-terminal sequence analysis of the pure bacteriocin obtained from reverse-phase HPLC showed that 48 amino acid residues had a sequence identical to the N-terminal sequence of carnobacteriocin B2 produced by \textit{C. piscicola} LV17B (33). The molecular mass calculated on the basis of the amino acid sequence was 4,969.5 Da. The bacteriocin produced by \textit{C. piscicola} A9b is called carnobacteriocin B2 from here on.

**Influence of inoculum size and addition of carbohydrate sources on bacteriocin production by \textit{C. piscicola} A9b in MR57—GCA broth at 25°C**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Glucose</th>
<th>Citrate</th>
<th>Acetate</th>
<th>Acetate + glucose</th>
<th>Acetate + citrate</th>
<th>Acetate + glucose + citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001% Inoculum</td>
<td>6.0</td>
<td>6.1</td>
<td>11.0</td>
<td>10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% Bac(^–) strain(^a)</td>
<td>0–0.2</td>
<td>0–0.2</td>
<td>4.8</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>1.6</td>
<td>1.1</td>
<td>11.5</td>
<td>11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>11.2</td>
<td>11.6</td>
<td>10.4</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + citrate</td>
<td>0–0.2</td>
<td>0–0.2</td>
<td>4.8</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate + glucose</td>
<td>10.0</td>
<td>10.0</td>
<td>9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate + citrate</td>
<td>11.6</td>
<td>11.6</td>
<td>12.0</td>
<td>10.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate + glucose + citrate</td>
<td>8.9</td>
<td>9.4</td>
<td>8.8</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
bacteriocin production was restored by the addition of a culture supernatant from the wild-type bacteriocin-producing culture (Bac/H11001) of strain A9b corresponding to an inoculum of 1.0%. A supernatant from the non-bacteriocin-producing mutant of A9b (Bac/H11002) did not influence the production of bacteriocin, a result which indicates that A9b Bac/H11001 produces an extracellular induction factor (Table 1).

The bacteriocin specific activity was doubled in the presence of acetate (increased from 600 to 1,000 BU/OD600 unit) and suppressed in the presence of glucose (decreased from 600 to 20 BU/OD600 unit) and citrate (decreased from 600 to 160 BU/OD600 unit). However, the suppressing effect of glucose and citrate on bacteriocin production was reversed by the addition of acetate or a filter-sterilized supernatant from the bacteriocin-producing culture (Bac/H11001) corresponding to an inoculum of 1.0% (Table 1). The level of production obtained in the presence of acetate and/or the supernatant from the Bac/H11001 culture was similar to that obtained when a 1.0% inoculum was used.

Characterization of the extracellular induction factor produced by C. piscicola A9b. The pattern of protease sensitivity of the extracellular induction factor produced by C. piscicola A9b was similar to that obtained for the bacteriocin, indicating that the induction factor could be the bacteriocin itself. The induction activity was recorded at each purification step of the bacteriocin except for reverse-phase HPLC. The induction factor was present in all fractions, (i) supernatant, (ii) ammonium sulfate precipitation, (iii) cation exchange, and (iv) HIC, corresponding to total induction activities of $3 \times 10^7$, $6.8 \times 10^7$, $3.3 \times 10^6$, and $2.1 \times 10^6$ IU ml$^{-1}$, respectively (data not shown). These data strongly indicate that carnobacteriocin B2 produced by C. piscicola A9b functions as an inducer of its own synthesis.

The induction activity was dose dependent up to a level of 20 IU of the induction factor ml$^{-1}$ in MRS7–CA broth (Fig. 1A), corresponding to a concentration of $1.9 \times 10^{-8}$ M (calculated from a protein concentration of $8.92 \times 10^{-8}$ mg ml$^{-1}$ and a size of 4,634 g mol$^{-1}$). At higher levels of the induction factor, the specific activity reached a plateau of approximately 800 BU/OD600 unit. The kinetics of the relationship in MRS7–CA broth could be described by a Michaelis-Menten equation. The critical concentration of carnobacteriocin B2 required to induce its own biosynthesis was $9.6 \times 10^{-10}$ M (calculated from a protein concentration of $4.46 \times 10^{-9}$ mg ml$^{-1}$ and a size of 4,634 g mol$^{-1}$), a value which corresponded to an approximate antilisterial activity of 0.007 BU.

The combination of acetate and increasing concentrations of carnobacteriocin B2 as an induction factor did not affect the saturation level of bacteriocin production (800 BU/OD600 unit) (Fig. 1A). However, the kinetics of the dose-dependent relationship in MRS7–CA broth differed from those in MRS7–CA broth, since the saturation level for bacteriocin production in MRS7–CA broth was reached at all levels of the induction factor carnobacteriocin B2 (from 0 to 100 IU ml$^{-1}$) (Fig. 1A).

Dose-dependent induction of bacteriocin production by acetate. The induction of bacteriocin production showed a dose-dependent relationship at acetate concentrations of up to 10 and 20 mM in MRS7–CA broth and APT broth, respectively (Fig. 1B). At higher acetate concentrations, the specific activity

![Graph A](http://aem.asm.org/content/110/11/2254/F1a)

![Graph B](http://aem.asm.org/content/110/11/2254/F1b)

**FIG. 1.** Dose-dependent induction of bacteriocin production in C. piscicola A9b by increasing concentrations of carnobacteriocin B2 and acetate as induction factors. (A) Carnobacteriocin B2 was added to MRS7–CA broth (▲) and MRS7–C broth (▼) at 25°C. One IU milliliter$^{-1}$ equals $9.6 \times 10^{-10}$ M induction factor. Data are from duplicate determinations. (B) Acetate was added to MRS7–CA broth (▲) and APT broth (▼) at 25°C. Data are from a single experiment.
reached plateaus of 900 to 1,200 BU/OD₆₀₀ unit and 120 BU/OD₆₀₀ unit in MRS7/H₁₁₀₀₂ CA broth and APT broth, respectively. In MRS7/H₁₁₀₀₂ CA broth, the plateau was unaffected by the addition of higher acetate concentrations of up to 90 mM, but the induction capacity of acetate was lost at approximately 70 mM acetate in APT broth, probably due to a low biomass. The kinetics of the relationship in MRS7/H₁₁₀₀₂ CA broth could be described by a Michaelis-Menten equation. The critical concentrations of acetate required to induce bacteriocin production were in the ranges of 0.3 to 1 mM and 12 mM in MRS7/H₁₁₀₀₂ CA broth and APT broth, respectively.

Influence of acetate on the induction of bacteriocin production. The production of bacteriocin by strain A⁹b in MRS7⁻C broth was detectable from an OD₆₀₀ of approximately 0.2 (Fig. 2). We hypothesized that bacteriocin production had to be induced before the culture reached this stage of growth (OD₆₀₀ ≈ 0.2). Acetate was added to the culture of A⁹b Bac⁺ at different stages in the growth phase (Fig. 2). Bacteriocin production reached a maximum level of 1,200 BU/ml when acetate was added within 8 h from the start of the experiment, corresponding to a cell density of less than approximately 5 × 10⁸ CFU/ml (Fig. 2). A minor reduction in production was observed when acetate was added after 10 to 12 h of incubation, and only small amounts of bacteriocin were detected when acetate was added after 14 h of incubation, corresponding to an OD₆₀₀ of approximately 0.2. At OD₆₀₀ of greater than 0.4 to 0.5, the addition of acetate did not result in detectable bacteriocin production (Fig. 2).

The induction capacity of acetate was investigated with several LAB strains. Acetate did not influence the induction of bacteriocin production in any of the other LAB strains investigated (data not shown).

Influence of glucose on the efficiency of acetate as an induction factor. The acetate concentration was determined during growth in MRS7⁻GCA broth and MRS7⁻CA broth to determine if glucose suppressed the production of acetate (Fig. 3). Bacteriocin production in MRS7⁻GCA broth reached a maximum concentration of 360 BU/ml in the stationary phase, whereas no bacteriocin was produced when glucose was added (in MRS7⁻CA broth) (Fig. 3A and B). Within the first 16 h of incubation, no significant differences in acetate concentration (Fig. 3D) or growth rate (Fig. 3A) were observed in comparison to the results obtained with MRS7⁻GCA broth. Although the concentration of glucose decreased by 15 mM (from 142.2 to 127.4 mM) within the first 16 h of incubation in MRS7⁻CA broth compared to only 0.1 mM (from 0.12 to 0.017 mM) in MRS7⁻GCA broth (Fig. 3C), no significant difference in pH was observed (Fig. 3E). These data indicate that the suppressing effect of glucose on the induction of bacteriocin production is not due to a decrease in the production of acetate or a decrease in the pH as a result of the metabolic conversion of glucose. However, from the present study, it is not clear whether the decrease in the pH in MRS7⁻CA broth (from 6.5 to 5.0) after the induction of bacteriocin production reduces the production of bacteriocin to low or undetectable levels.

We hypothesized that glucose may interfere with the response to acetate as an induction factor. To test this hypothesis, acetate and glucose were added to MRS7⁻GCA broth at
different concentrations (Fig. 4). The induction efficiency of acetate decreased with increasing concentrations of glucose. However, glucose had no apparent effect on the induction efficiency of acetate at a high concentration (36.74 mM). In media with low acetate concentrations (1.8 and 5.5 mM), the addition of even small amounts of glucose (from 13.9 mM) reduced the induction efficiency of acetate dramatically (from approximately 350 BU/ml to 0 to 45 BU/ml).

Influence of food-relevant environmental factors on the induction capacities of acetate and carnobacteriocin B2. The growth of *C. piscicola* in cold smoked salmon juice caused an increase in the acetate concentration from 3 to 5 mM to 9 mM in 32 days at 5°C and a decrease in the glucose concentration from 4 mM to 0.5 mM in 20 days. Since the concentration of acetate was above the threshold level for induction in MRS7–CA broth (0.3 to 1 mM) and the concentration of glucose was low, we hypothesized that acetate could act as a natural induction factor in cold smoked salmon juice. To test this hypothesis, acetate was added to cold smoked salmon juice (with 4 and 5% [wt/vol] NaCl) at increasing concentrations ranging from 0 to 36.74 mM. No bacteriocin production was found in any of the samples (data not shown), even in the presence of a high acetate concentration (Table 2). These data indicate that acetate could not act as an inducer for bacteriocin production in cold smoked salmon juice.

The induction capacity of acetate decreased dramatically with increasing concentrations of salt in MRS7–CA broth, and no induction effect was observed in the presence of 1.5% NaCl (Table 2). The induction capacity of carnobacteriocin B2 was not influenced by the presence of 0 to 2.5% NaCl in MRS7–CA broth but was decreased in the presence of 3.0% NaCl (reduced from 640 to 320 BU ml⁻¹) (Table 2) to 7.0% NaCl (reduced from 640 to 20 BU ml⁻¹) (Table 2).

The induction capacity of carnobacteriocin B2 in cold smoked salmon juice in the presence of 4% NaCl was investigated. Bacteriocin was induced by carnobacteriocin B2, and bacteriocin activity was similar to that obtained in MRS7–CA broth with 4% NaCl. However, the critical level of carnobacteriocin B2 needed to induce its own biosynthesis at 25°C was approximately 450 times higher in salmon juice with 4% NaCl than in MRS7–CA broth without NaCl (increased from 9.6 \times 10^{-10} to 4.3 \times 10^{-7} M) (data not shown).

We tested the antilisterial effect of *C. piscicola* A9b in a coculture with *L. monocytogenes* in cold smoked salmon juice in the absence and presence of the induction factor carnobacteriocin B2 (Fig. 5). The number of *L. monocytogenes* cells as a pure culture increased from 10³ to 10⁶ CFU/ml after 22 days of storage at 5°C. The growth of *L. monocytogenes* was unaffected in the mixed culture with *C. piscicola* A9b (initial level of 10⁸ CFU/ml) when the induction factor was not added. In the absence of the induction factor, no bacteriocin was produced during storage. However, in the presence of the induction factor (114 IU ml⁻¹), the growth of *L. monocytogenes* was

**FIG. 3.** Growth of *C. piscicola* A9b Bac⁻¹ in MRS7–GCA broth (□) and MRS7–CA broth (■) at 25°C. OD (A), bacteriocin production (B), glucose concentration (C), acetate concentration (D), and pH (E) were determined. The error bars indicate means and standards deviations of duplicate determinations.
suppressed after 17 days of incubation and the maximum cell number was reduced by 2.5 log units. The inhibition of *L. monocytogenes* coincided with the detection of produced bacteriocin. The growth of *L. monocytogenes* was strongly reduced in the presence of a high initial cell number of *C. piscicola* (10⁶ CFU/ml) and in the presence of the induction factor carnobacteriocin B2 (114 IU ml⁻¹). Bacteriocin was detected after 6 days of incubation, and the level increased from 80 BU ml⁻¹ initially to 320 BU ml⁻¹ at the end of storage. A higher concentration of added inducer (457 IU ml⁻¹) resulted in the production of bacteriocin after 2 days of incubation and an enhanced inhibition of *L. monocytogenes* (data not shown).

**DISCUSSION**

*C. piscicola* strain A9b was originally isolated as part of the dominant flora of cold smoked salmon (29) and used as a bioprotective culture for inhibiting *L. monocytogenes* in cold smoked salmon (27). In this study, we demonstrated that *C. piscicola* A9b produces an autoinducible antilisterial bacteriocin that is induced by acetate. To our knowledge, this is the first time that acetate has been described as an induction factor for bacteriocin production.

The N-terminal amino acid sequence of the bacteriocin produced by *C. piscicola* A9b was identical to that of carnobacteriocin B2 from *C. piscicola* LV17B (32, 33), which also produces carnobacteriocins A and BM1. The bacteriocin produced by *C. piscicola* A9b is therefore referred to here as carnobacteriocin B2. Carnobacteriocin B2 from LV17B is encoded on a 61-kb plasmid, and BM1 is encoded from a genetic fragment (1, 33). *C. piscicola* A9b produces only carnobacteriocin B2, and the genetic background differs from that of *C. piscicola* LV17B. It frequently happens that the same bacteriocin is isolated from several strains of the same species, e.g., lactobin A and amylovorin L471 (6), carnobacteriocin BM1 and piscicocin V1b (5), piscicocin V1a and piscicocin 126 (12), and mesenterocin 52A and mesentericin Y105 (36).

Our purification procedure consisted of four steps; in contrast, Quadri et al. (33) purified bacteriocins B2 and BM1 by a three-step procedure. Our last step, reverse-phase chromatography, was used only for desalting purpose but resulted in a 35-fold increase in specific activity.

Bacteriocin production in LAB can be influenced by environmental parameters, which may act on the bacteriocin regulatory system (9, 12) or affect the binding of the induction factor to its receptor (25). In this study, we have demonstrated that in MRS broth with glucose as a C source, acetate is required to induce bacteriocin production in *C. piscicola* A9b. In other studies, growth and bacteriocin production of *C. piscicola* strains were obtained in MRS broth without acetate (39,
A large inoculum (0.5%) may explain why bacteriocins were produced in these studies. No similar effect of acetate was observed in any other examined bacteriocin-producing LAB. Bacteriocin production is stimulated by less favorable growth conditions, such as low temperature (23), a competitive flora (41), high osmolarity (NaCl) (43), ethanol (23), or mitomycin C (19). It has been suggested that bacteriocin production could be the result of the transcription of genes involved in stress conditions (19). It has been suggested that bacteriocin production could be the result of the transcription of genes involved in stress conditions (19).

Acetate induction has several features in common with peptide-based induction of bacteriocin production in two- or three-component signal transduction systems. Acetate must be present during the early exponential growth phase for induction to occur, as is the case for most peptide-based inducers regulating bacteriocin production, such as those in C. piscicola LV17B (38) and E. faecium CTC492 (25). The acetate induction of bacteriocin was dose dependent, and increasing NaCl or glucose concentrations reduced the efficiency of acetate as an inducer. These relationships are similar to those for the peptide-based induction factor in E. faecium CTC492 (25), where it has been suggested that NaCl negatively influences the binding of the induction factor to its receptor (25).

We found that glucose repressed the induction efficiency of acetate for carnobacteriocin production (Fig. 4). This result could have been caused by a direct effect of glucose on bacteriocin production or indirectly through changes in pH. Glucose can negatively affect response regulators; e.g., in Staphylococcus aureus, glucose reduces the expression of the accessory gene regulator (agr), which regulates the expression of numerous exoproteins in two-component signal transduction systems (35). Further, pH is also known to influence the regulation of signal transduction systems (25, 34). The inducing effect of acetate occurred before changes in pH or acetate concentrations could be measured. We therefore suggest that glucose by itself repressed the induction efficiency of acetate.

We also demonstrated that the biosynthesis of carnobacteriocin B2 in C. piscicola strain A9b is an autoinduced process similar to B2 and BM1 production in C. piscicola LV17B (32, 38) and to the production of some lantibiotics, e.g., nisin (17). The autoinduction is dose dependent, like that for bacteriocin production in E. faecium CTC492 (25).

A threshold concentration of carnobacteriocin B2 from A9b of approximately 10^{-9} M was required to trigger its own biosynthesis. This level is similar to the threshold level of about 10^{-10} M previously reported for nisin (17), sakacin P (11), and carnobacteriocins B2 and BM1 (32) but significantly higher than the level of 10^{-17} M found for induction factor EntF in E. faecium (25). The highest yield (800 to 1,200 BU ml^{-1}) was unaffected by the type of induction factor (acetate or bacteriocin) present, and Nilsen et al. (25) similarly showed that bacteriocin production reaches a saturation level. The production of carnobacteriocin B2 from strain A9b was not enhanced by the combined presence of both induction factors and, to our knowledge, this is the first time that the combined action of two induction factors on bacteriocin production has been examined.

Acetate is present in freshly produced dry salted cold...
smoked salmon at levels of 2 to 3 μmol g⁻¹ (42), and levels increase to approximately 23 μmol g⁻¹ after 25 days of storage at 5°C as a result of microbiological growth (42). However, in the present study, acetate did not induce bacteriocin production in cold smoked salmon juice due to the presence of moderate NaCl levels (3 to 5% water-phase salt). The induction capacity of acetate was lost at NaCl concentrations above 1.5% in MR57–CA broth. Increasing NaCl concentrations also reduced the efficiency of carnobacteriocin B2 from strain A9b as an induction factor, but induction did occur, albeit at a lower efficiency, even at a NaCl concentration of 7%. Nilsen et al. (25) also showed that NaCl negatively influenced the induction efficiency of EntF; at 6.5% NaCl, 300 times more EntF was needed to induce detectable bacteriocin production (25). In cold smoked salmon juice, however, adding carnobacteriocin B2 as an induction factor restored bacteriocin production, and detection coincided with the inhibition of L. monocytogenes. It was suggested that NaCl negatively influences the binding of the induction factor to its receptor (25).

In conclusion, this study we have demonstrated that although both acetate and carnobacteriocin induce the production of carnobacteriocin B2 in strain A9b, only the bacteriocin itself can be used to manipulate the production of bacteriocin in cold smoked salmon juice and thereby improve the ability of C. piscicola strain A9b to inhibit L. monocytogenes. Further, high initial levels (e.g., 10⁶ CFU/g) of the bacteriocinogenic culture must be used when bacteriocin production does not occur before the middle to late exponential phase of growth, as occurs in C. piscicola.

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