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The contribution of bacteriocin to inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* strains in cold-smoked salmon systems

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ABSTRACT

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Aims: To study the importance of bacteriocin production for the antilisterial effect of a bacteriocinogenic *Carnobacterium piscicola* strain A9b on growth of *Listeria monocytogenes* in broth and cold-smoked salmon systems.

Methods and Results: Acriflavin treatment of strain A9b resulted in loss of bacteriocin production and of immunity to carnobacteriocin B2. Two plasmids present in the wild-type were lost in the variant that was also more sensitive to bavaricin and leucocin A than the wild-type indicating cross-resistance to class IIa bacteriocins. The growth rate of the bac$^-$ mutant was higher than that of the wild-type at 5 and 37°C but not at 25 or 30°C. In salmon juice the maximum cell density of *L. monocytogenes* was suppressed 3 and 6 log by co-culture with *C. piscicola* A9b bac$^-$ and bac$^+$, respectively, as compared with the control. Sterile filtered cultures of *C. piscicola* A9b bac$^-$ caused a limited suppression of the maximum cell density of *L. monocytogenes* similar to that observed when sterile buffer was added in equal amounts. Semi-purified carnobacteriocin B2 caused a 3·5 log decline in viable cell count after 6 day of incubation in cold-smoked salmon juice at 5°C. High resistance level to carnobacteriocin B2 was observed for *L. monocytogenes* cells exposed to semi-purified and *in situ* produced carnobacteriocin B2.

Conclusions: The presence of bacteriocin production in *C. piscicola* enhances its inhibition of *L. monocytogenes*

Significance and Impact of the Study: Due to the emergence of resistance, a bacteriocin negative lactic acid bacteria may be more suited for practical use as a bioprotective agent against *L. monocytogenes* in ready-to-eat foods.

Keywords: antagonism, carnobacteriocin B2, *Carnobacterium piscicola*, cold-smoked salmon, *Listeria monocytogenes*, plasmid.

INTRODUCTION

During the last several decades, research effort directed towards preservation strategies of (micro)biological origin has flourished. In particular, the use of lactic acid bacteria (LAB) and/or their bacteriocins for use in biopreservation of foods is an area of great interest. Several bacteriocins have been identified and characterized and the search for new antimicrobial peptides is still intense although the applicability of bacteriocins in food manufacturing is being questioned (Mazotta *et al.* 1997; Nes *et al.* 2002; Ryan *et al.* 2002). In food matrices bacteriocins are strongly absorbed to food components, and the solubility and stability is often poor due to their physiochemical properties (Gänzle *et al.* 1999). As a consequence, the actual bacteriocin activity in the environment of the bacteria is much lower than expected (Nes *et al.* 2002). Other factors that limit use of bacteriocins include resistance development among target bacteria (Harris *et al.* 1991; Ming and Daeschel 1993; Mazotta *et al.* 1997;

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Crandall and Montville 1998; Nilsson *et al.* 2000). Further, bacteriocin production by LAB is often inconsistent and low in food products, possibly because of repression of bacteriocin synthesis. The synthesis of many bacteriocins is regulated by a two- or three-component signal transduction system, which includes induction by the bacteriocin itself (autoinduction) or by an extracellular secreted peptide. However, the secretion and efficiency of the signal molecule, which trigger the transcription of genes coding for bacteriocin production, is affected by environmental factors such as pH, presence of ethanol and sodium chloride and may be turned off in some food systems (Nes *et al.* 2002). Recently it was demonstrated that these problems may partly be overcome by addition of the induction factor (Nilsen *et al.* 1998; Nilsson *et al.* 2002).

The use of bacteriocins and/or bacteriocin-producing strains for biopreservation in the food industry is only viable if the limiting factors mentioned above can be overcome. Therefore, it is of interest to focus more on nonbacteriocin-producing organisms as bioprotective cultures. It must be emphasized that several studies have demonstrated that nonbacteriocinogenic LAB have acid-independent antibacterial properties (Degnan *et al.* 1992; Leroi *et al.* 1996; Buchanan and Bagi 1997; Nilsson *et al.* 1999). Previously we demonstrated that both a bacteriocin-producing strain (*Carnobacterium piscicola* strain A9b) and a nonbacteriocin-producing strain (*C. piscicola* strain A10a) were effective in controlling the growth of *L. monocytogenes* in cold-smoked salmon (Nilsson *et al.* 1999). Therefore it was hypothesized that the ability of *C. piscicola* A9b to produce bacteriocin on cold-smoked salmon did not play a major role in controlling the growth of *L. monocytogenes*. However, the strains compared were not isogenic and therefore the importance of bacteriocin production *per se* could not be addressed. To further test this hypothesis we have, in the present study, compared the antilisterial effect of the wild-type *C. piscicola* A9b (bac^+) and its nonbacteriocin-producing mutant (bac^-) in laboratory media, in model fish system and in vacuum-packed cold-smoked salmon. We demonstrate that *C. piscicola* A9b bac^- inhibits *L. monocytogenes* and that a significant nonbacteriocin-dependent inhibition is functioning.

MATERIALS AND METHODS

Bacterial strains, culture conditions and media

The bacteriocin producer *C. piscicola* strain A9b was isolated from vacuum-packed cold-smoked salmon (Paludan-Müller *et al.* 1998). *Listeria monocytogenes* strain O57 (Ben-Embarek and Huss 1993) and *C. piscicola* DSM 20730 (obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were used as an indicator organisms for bacteriocin quantification in a well diffusion assay. *Carnobacterium piscicola* DSM 20730 was used as indicator organism to measure bacteriocin production in salmon juice experiments and in cold-smoked salmon, where only small amounts of bacteriocin were produced. The class IIa bacteriocins were prepared as fermentations of the producer organisms: *Lactobacillus sake* MI401 (Larsen *et al.* 1993) (reclassified from *Lactobacillus bavaricus*) producing bavaricin, *Leuconostoc gelidum* UAL 187-22 (Papathanasopoulos *et al.* 1997) producing leucocin A and *Pediococcus acidilactici* PA-2 producing PA-1 (Chr. Hansen A/S, Hørsholm, Denmark). Further *Lactococcus lactis* was used for the production of nisin. *Escherichia coli* V517 (Macrina *et al.* 1978), *C. piscicola* LV17 (Ahn and Stiles 1990; Quadri *et al.* 1994) and *C. divergens* LV13 (Worobo *et al.* 1995) were used as molecular mass reference standards for plasmid size.

Carnobacterium piscicola and *L. monocytogenes* strains were propagated twice in BHI (Brain Heart Infusion) broth (Oxoid CM225) at 25°C for 18 h. *Lactobacillus sake* and *L. lactis* were grown in MRS (deMan, Rogosa and Sharpe) broth (E. Merck AG, Damstadt, Germany, 1.10661) at 30°C for 18 h. *Escherichia coli* was propagated twice in LB (Luria–Bertani, Difco 244620) broth at 37°C. Working cultures were maintained at 5°C on BHI agar (BHI broth containing 1·2% agar, code BBB10030, Bie & Berntsen A/S, Højbjerg, Denmark), MRS agar (Oxoid, CM361) or LB agar, and frozen stock cultures were stored at −80°C. When required, BHI broth was buffered (B-BHI) with 0·1 N phosphate buffer (K_2HPO_4/KH_2PO_4) to pH 6·2. MRS broth adjusted to pH 7·0 before autoclaving (MRS7) was used as growth medium for maximal bacteriocin production (Himelbloom *et al.* 2001).

Cold-smoked salmon juice was prepared (Nilsson *et al.* 1999). In brief, freshly produced cold-smoked salmon was mixed with tap water in a 2 : 1 ratio and boiled for 10 min. Salt analysis was performed (AOAC 1975) and NaCl was added to a final concentration of 4·0% (wt/vol). The juice was heated for 30 min at 100°C and stored in the dark at 5°C for a maximum of 3 days before use.

Selection and characterization of a bac^- mutant

Plasmid curing was performed by growing *C. piscicola* A9b bac^+ in BHI broth (1% inoculum) containing 20 $\mu g\ ml^{-1}$ acriflavin (Sigma A-8126) at 25°C. Three consecutive transfers in presence of acriflavin were performed. When growth was visible, appropriate dilutions were spread on BHI agar at 25°C for 24 h. Regenerated colonies (six in total) were screened for bacteriocin production against the indicator species *L. monocytogenes* O57 and *C. piscicola* DSM 20730 in an agar diffusion assay (Nilsson *et al.* 1999; see description below). Lack of clearing zones from acriflavin-exposed clones were indicative of loss of

bacteriocin production and one such bacteriocin-negative colony was selected for further studies. To confirm that the variant was indeed originating from strain A9b, random amplified polymorphic DNA (RAPD) patterns of the two strains were compared. Preparation of DNA and RAPD conditions followed Fonnesbech Vogel *et al.* (2001) and primers used were UBC155 (5′-CTGGCGGCTG), OMP1 (5′-GTTGGTGGCT) and DAF4 (5′-CGG-CAGCGCC) all from DNA-Technology, Aarhus, Denmark. Physiological and genetic properties of the wild-type and bacteriocin-negative mutant of *C. piscicola* A9b were compared: (i) pattern of carbohydrate fermentation (API 50 CH; bioMérieux, Lyon, France, code 50300 and 50410), (ii) growth rate of cultures at 5, 25, 30 and 37°C in BHI broth, (iii) bacteriocin production at 25, 30 and 37°C in MRS7, (iv) inhibition of *L. monocytogenes* O57 and *C. piscicola* DSM 20730 by sterile-filtered supernatant and culture precipitates in an agar diffusion assay, (v) SDS–PAGE analysis of precipitate, (vi) plasmid profiles of cultures, (vii) sensitivity to bacteriocin from *C. piscicola* A9b (carnobacteriocin B2), *Lactobacillus sake* MI401 (bavaricin), *Pediococcus acidilactici* PA-2 (pediocin PA-1), *Leuconostoc gelidum* UAL 187-22 (leucocin A) and *L. lactis* (nisin) in an agar diffusion assay.

Plasmid isolation

Minipreparations of plasmid DNA from *C. piscicola* A9b were obtained by a modification of the Klaenhammer (1984) method. In brief, 3 ml of an overnight culture grown in BHI at 25°C was centrifuged at 12 000 *g* for 5 min and washed in 1 ml of cold 0·5% NaCl. Cells were resuspended in 100 μl of a lysozyme (Sigma) solution (10 mg ml^{-1} in 25% (wt/vol) sucrose in 50 mmol l^{-1} Tris–HCl, 5 mmol l^{-1} Na_2 EDTA, pH 8·0), incubated at 37°C for 1 h and mixed with 200 μl 0·9% glucose in 0·2 N NaOH, 50 mmol l^{-1} Tris buffer containing 5 mmol l^{-1} EDTA and 3% SDS. After mixing, 50 μl of 2 M Tris (pH 7·0) and 70 μl 5 M NaCl was added and the vial left for 5 min. Deproteinization of the sample was conducted with 3% NaCl-saturated phenol (Sigma P-4557) and chloroform : isoamylalcohol was added to facilitate phase separation. The aqueous phase was mixed with 1 ml of 95% ethanol and held at −20°C overnight. DNA was pelleted by centrifugation at 12 000 *g* for 15 min at 5°C and subjected to 0·3 and 0·7% agarose (15510–019 Life Technologies, Paisley, UK) gel electrophoresis in TAE buffer (40 mmol l^{-1} Tris acetate, 1 mmol l^{-1} EDTA, pH 8·0) at 90–120 V for 2·5–4 h. Plasmid sizes were estimated by using *E. coli* V517 (Macrina *et al.* 1978), *C. divergens* LV13 (Worobo *et al.* 1995) and *C. piscicola* LV17 (Ahn and Stiles 1990), which contains plasmids of known sizes. Plasmids were extracted from these three strains following the procedure described above.

Bacteriocin assay

The bacteriocin concentration was determined in an agar diffusion assay as previously described (Nilsson *et al.* 1997). *Listeria monocytogenes* O57 was used as indicator organism, but when no bacteriocin was detected a more sensitive target strain (*C. piscicola* DSM 20730) was used for verification. The indicator organism was diluted to 10^6 CFU ml^{-1} in 48°C BHI agar (BHI broth containing 0·8% agar) supplemented with 0·1% Tween-80 (Merck, 822187) and poured into 14 cm Petri dishes (Sterilin, Bibby, Sterilin Ltd, Stone, Staffs, UK). Supernatant fluid from the producer strain *C. piscicola* strain A9b was filter sterilized (0·2 μm pore size nylon membrane filter, Sartorius, 16534K). Samples (50 μl) were pipetted into wells with a 7 mm diameter cut into the agar as described by Nilsson *et al.* (1997). The plates were preincubated at 5°C for 24 h followed by 18 h at 25°C or 4–6 h at 37°C for *L. monocytogenes* O57 and *C. piscicola* DSM 20730, respectively. Concentration of bacteriocin was assayed by testing twofold dilutions series in 0·01 mol l^{-1} K_2HPO_4/KH_2PO_4 (pH 6·0) of supernatant fluid as previously described by Nilsson *et al.* (1997). 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 50 μl, and BU per millilitre was calculated.

Preparation of precipitated bacteriocin from *C. piscicola* A9b bac^+ and its bac^- mutant

Bacteriocin produced by *C. piscicola* A9b bac^+ in MRS7 broth (MRS broth adjusted to pH 7·0 (Himelbloom *et al.* 2001) was precipitated by ammonium sulphate (Merck, code 1.01217) and desalted by dialysis as previously described (Nilsson *et al.* 2002). A similar precipitate was also prepared from *C. piscicola* A9b bac^-. Bacteriocin activity was determined using *L. monocytogenes* O57 and *C. piscicola* DSM 20730 as indicator organisms. Proteins in the precipitated supernatants were separated by SDS–PAGE using 12% NuPAGE Bis–Tris gels (Novex, San Diego, CA, USA) as previously described (Nilsson *et al.* 2002).

Determination of carnobacteriocin B2 resistance

Listeria monocytogenes colonies isolated from Listeria selective agar were inoculated and grown in BHI at 30°C for 18 h reaching approx. 5×10^8 CFU ml^{-1}. Fresh BHI broth was inoculated to approx. 10^6 cells ml^{-1} and pipetted into the wells of a microtitre plate (Nunc 64122, Roskilde, Denmark). Carnobacteriocin B2 obtained from precipitated supernatant of *C. piscicola* A9b bac^+ (corresponding to approx. 10 000 BU ml^{-1}) was added in the first row of wells. Twofold dilutions of carnobacteriocin B2 in the culture were

made, and the minimum inhibitory concentration (MIC) was determined as the lowest concentration carnobacteriocin B2 which prevented *L. monocytogenes* growth after 24 h at 30°C. This MIC was compared with the MIC of the isolate grown without *Carnobacterium* or bacteriocin. Resistance was defined if the MIC of the exposed isolate was higher than that of the nonexposed isolate.

Interaction between *L. monocytogenes* and *C. piscicola* A9b bac$^+$ and its bac$^-$ mutant in laboratory media

The importance of bacteriocin production by *C. piscicola* A9b for inhibition of *L. monocytogenes*, and the influence of the initial cell number of both *L. monocytogenes* O57 and *C. piscicola* A9b bac$^+$ and bac$^-$ on the growth of the cultures were investigated in B-BHI broth with 4% NaCl (wt/vol) at 5°C. All cultures were preincubated as monocultures at 15°C in B-BHI broth supplemented with 3% NaCl until cells reached the late exponential phase of growth (O.D.$_{600\ nm}$ of 0·6–0·7, Novaspec. II, Amersham Pharmacia Biotech., Hillerød, Denmark). Cells were centrifuged (6000 *g* for 15 min, 5°C), washed once in 0·1% sterile peptone water, and finally resuspended in 0·1% peptone water to appropriate cell numbers. The harvesting and washing step was included to prevent carry-over of preformed bacteriocin to the co-culture experiment. *Carnobacterium piscicola* A9b bac$^+$ and *L. monocytogenes* O57 or *C. piscicola* A9b bac$^-$ and *L. monocytogenes* O57 were inoculated as mono and mixed strains at concentrations of 10^3 or 10^6 CFU ml^{-1}. All cultures were prepared in duplicate. Samples were removed from the cultures once or twice a week for the determination of colony counts and bacteriocin production.

Mono and co-cultures of *L. monocytogenes* and *C. piscicola* A9b bac$^-$ were also grown in B-BHI broth at 30°C. Cultures were preincubated at 30°C until cells reached the mid-exponential phase of growth (O.D.$_{600}$ of 0·4–0·5). Cells were washed as described above before inoculation corresponding to a level of 10^3 and 10^6 CFU ml^{-1} of *L. monocytogenes* and *C. piscicola* A9b bac$^-$, respectively.

Listeria monocytogenes was enumerated by direct plating of 0·1 ml of appropriate dilutions onto listeria-selective agar base (Oxoid, CM856), to which listeria selective supplement (Oxoid, SR206E) was added. The plates were incubated at 25°C for 48 h. Cell numbers of *C. piscicola* were estimated by direct plating onto nitrate polymyxin agar (NP, composed as nitrite actidion polymyxin agar without actidion) made from APT agar (Difco, 0654-17), pH 6·7 (Davidson and Cronin 1973). Plates were incubated at 25°C for 48 h. Pinpoint (<1 mm) colonies were not counted as these were found to be *L. monocytogenes* capable of weak growth on NP agar.

Inhibition of *L. monocytogenes* O57 in salmon juice and on cold-smoked salmon by *C. piscicola* A9b bac$^+$ and its bac$^-$ mutant

Carnobacterium piscicola A9b was precultured in APT broth (1% preculture) supplemented with 4·0% NaCl (wt/vol) to exponential phase of growth (5 days at 5°C). Cells were harvested and washed as described above. *Listeria monocytogenes* O57 was grown in BHI broth supplemented with 3% NaCl (1% preculture) to exponential phase (24–26 h at 15°C) and diluted in 0·1% peptone water to appropriate cell numbers.

Cold-smoked salmon juice with 4·0% NaCl (wt/vol) was inoculated with *L. monocytogenes* O57 to a cell density of 10^4 CFU ml^{-1}. *Carnobacterium piscicola* A9b bac$^+$ and bac$^-$ was added to a final concentration of approximately 10^6 CFU ml^{-1}. Precipitated bacteriocin from stationary cultures of *C. piscicola* A9b bac$^+$ and bac$^-$ were added to some of the flasks at a concentration of 1%. The flasks were incubated for 32 days at 5°C in the dark. Samples were removed once or twice a week for colony counts, bacteriocin activity and resistance to carnobacteriocin B2 (see description above). All trials were carried out in duplicate.

Cold-smoked salmon was obtained from a commercial producer. The salt content was determined (AOAC 1975). Slices were prepared aseptically and inoculated with approx. 10^3 CFU g^{-1} *L. monocytogenes* and 10^6 CFU g^{-1} *C. piscicola* A9b bac$^+$ or bac$^-$. Controls included slices inoculated with *L. monocytogenes* alone. Slices were vacuum-packed (Riloten/× 40/50 Conovac, Otto5 Nielsen Emballage A/S, Lyngby, Denmark; permeability for O_2-1·0 cm^3 m^{-2} and CO_2-4·0 cm^3 m^{-2} in 24 h, 1 atm at 23°C, 95% relative humidity) and stored at 5°C. At regular intervals during storage, samples were withdrawn, diluted 1 : 10 in sterile saline and homogenized using a stomacher. Tenfold serial dilutions were made and *L. monocytogenes* and *C. piscicola* enumerated as described above. Two packages were sampled on each sampling day and results presented as mean of log-transformed counts.

RESULTS

Characterization of a bac$^-$ mutant

Six colonies were randomly picked from *C. piscicola* exposed to acriflavin. The strains were pure cultured and tested for inhibitory activity against *L. monocytogenes*. A culture lacking inhibitory activity was grown in BHI broth and the supernatant sterile-filtered or precipitated with ammonium sulphate. Neither of these preparations inhibited *L. monocytogenes* O57 or *C. piscicola* DSM 20730 in the well diffusion assay (data not shown). The 4·9 kDa molecular weight bacteriocin (carnobacteriocin B2) (Nilsson *et al.* 2002) was not present in the culture precipitate of the

mutant when assayed by SDS–PAGE of the precipitated bac$^-$ culture (data not shown).

The bac$^-$ mutant and the wild-type strain had identical patterns of carbohydrate fermentation in the API system and the two strains were identical by RAPD-typing using the three primers UBC155, OMP1 and DAF4 (data not shown). The growth rate of the bac$^-$ mutant was higher than that of the wild-type at 5 and 37°C whereas growth rate at 25°C was similar (Fig. 1). The growth rates at 30°C was identical to that at 25 (data not shown). The wild-type strain of A9b produced bacteriocin in MRS7 at 25, 30 and 37°C corresponding to levels of 1280, 1280 and 40 BU ml^{-1}, and no bacteriocin activity was detected in the bac$^-$ mutant.

The plasmid profile of the wild-type strain of *C. piscicola* A9b was difficult to resolve (Fig. 2). The strain contained two distinct plasmid bands of approx. 67 and 47 kb on the basis of multiple regression calibration with plasmids from *E. coli* V517 and *E. coli* 39R. The bacteriocin-negative mutant of the wild-type had lost the 67- and 47-kb plasmids. Based on the present data we cannot conclude if bacteriocin production is coupled to both of these plasmids or if the appearance of two bands in the agarose is caused by one (the 67 kb) being an open circular form of the other (47 kb) plasmid. This observation was tested by isolation of plasmid DNA and comparison of digestion patterns. Under u.v. light the bands were cut and re-extracted by methods described by McDonell *et al.* (1977) using a gel band purification kit (Amersham Pharmacia Biotech., 27-9602-01). However re-extraction of plasmid DNA was unsuccessful due to a very low recovery and consequently digestion patterns were never analysed. Therefore, it was not possible to conclude if bacteriocin production was linked to two plasmids in this organism.

The bac$^-$ mutant was sensitive to carnobacteriocin B2. Also, the bac$^-$ mutant displayed increased sensitivity to the class IIa bacteriocins bavaricin and leucocin A as compared with the bac$^+$ wild-type (Table 1). Neither the wild-type and bac$^-$ mutant were immune to pediocin PA-1 and nisin (Table 1).

Interaction between *L. monocytogenes* and *C. piscicola* A9b bac$^+$ and its bac$^-$ mutant in laboratory media

The cell number of *L. monocytogenes* as pure culture increased from 10^4 CFU ml^{-1} to a maximum of 10^9 CFU ml^{-1} after 12 days of storage in B-BHI broth with 4·0% NaCl at 5°C (Fig. 3a). The presence of a high initial cell number of *C. piscicola* bac$^+$ (10^6 CFU ml^{-1}) resulted in a 2-log reduction of viable listeria count after 8 days of incubation as compared with the control. The inhibition of *L. monocytogenes* coincided with the detection of bacteriocin. The growth of *L. monocytogenes* was also influenced by co-culture with *C. piscicola* A9b bac$^-$ and cell numbers were reduced with 1–1·5 log after 8 days and with

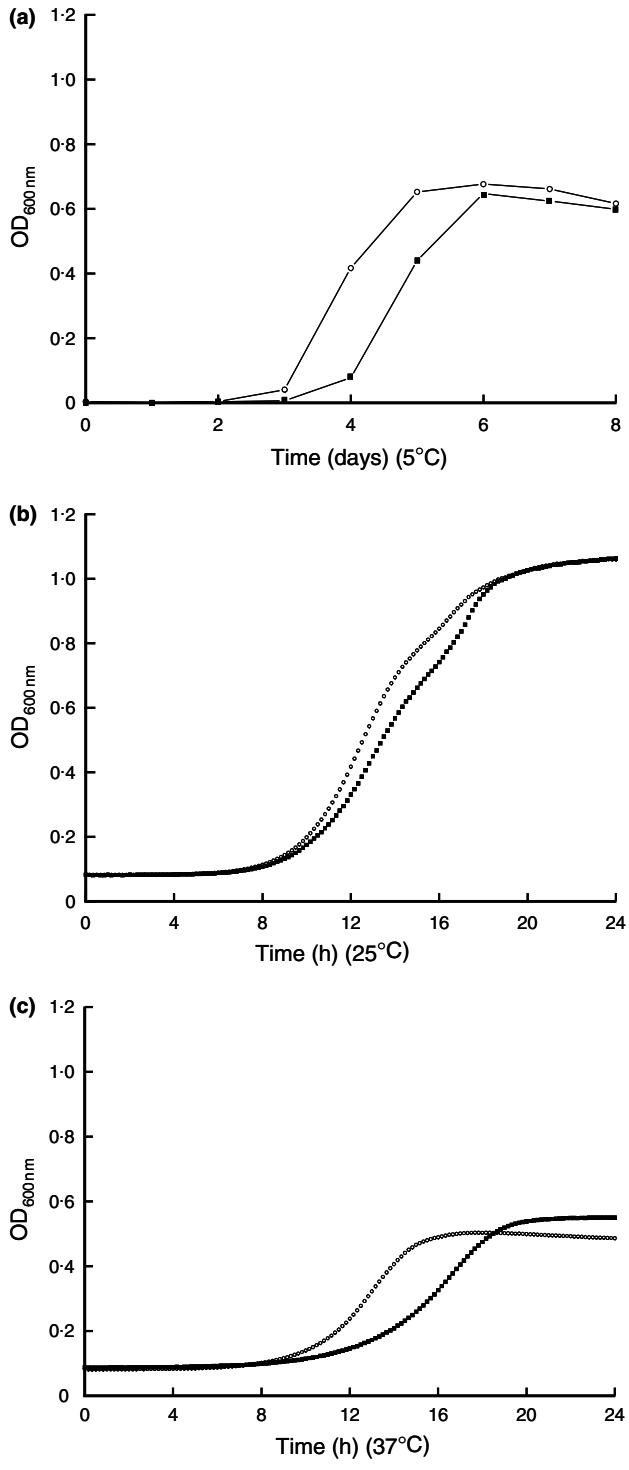

Fig. 1 Growth of *Carnobacterium piscicola* A9b bac$^+$ and bac$^-$ cultures in buffered BHI broth at 5°C (a), 25°C (b) and 37°C (c). *C. piscicola* A9b bac$^-$ (○) and *C. piscicola* A9b bac$^+$ (■). Growth was determined at an O.D.$_{600}$ in microtitre plates, the O.D. values were normalized to a path length of 1 cm

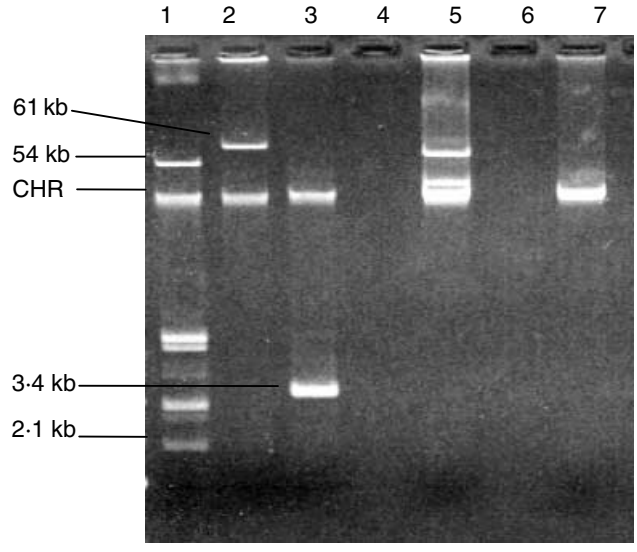

Fig. 2 Agarose gel electrophoresis of plasmid DNA from *Carnobacterium piscicola* A9b. Bands are identified top to bottom. Lane 1: *E. coli* V517 54, 7·4, 5·6, 5·1, 4·4, 3·0, 2·7 and 2·1 kilobases covalently closed circular DNAs; lane 2: *C. piscicola* LV17 (74 and 61 kb); lane 3: *C. divergens* LV13 (3·4 kb); lane 4: empty; lane 5: bacteriocin-positive variant of *C. piscicola* A9b; lane 6: empty; lane 7: bacteriocin-negative vartiant of *C. piscicola* A9b (parental strain). The molecular mass of plasmids are indicated. CHR, chromosomal DNA

Table 1 Activities of class IIa bacteriocins towards wild-type strain and a bac$^-$ mutant of *Carnobacterium piscicola* A9b

		Sensitivity (MIC, BU ml^{-1}) of *C. piscicola* A9b	
Strain	Bacteriocin	Wild-type	bac$^-$ mutant
Lactobacillus sake MI401	Bavaricin	16	1
Leuconostoc gelidum UAL 187-22	Leucocin A	128	2
Pediococcus acidilactici PA-2	Pediocin PA-1	16	8
Lactococcus lactis	Nisin	4	4
Carnobacterium piscicola A9b	Carnobacteriocin B2	256	4

3 log after 38 days as compared with the control (Fig. 3a). A 3·5 log reduction in the maximum cell density was observed at 30°C (data not shown). Cell-free supernatants of *C. piscicola* A9b bac$^-$ were not inhibitory to *L. monocytogenes*, neither when derived from a monoculture nor from a co-culture with *L. monocytogenes* (data not shown).

Carnobacterium piscicola A9b bac$^+$ and bac$^-$ grew rapidly as pure cultures in B-BHI broth with 4% NaCl at 5°C, increasing from 2×10^6 CFU ml^{-1} to maximum counts of *ca* 5×10^8 CFU ml^{-1} after 8 days of incubation (Fig. 3b).

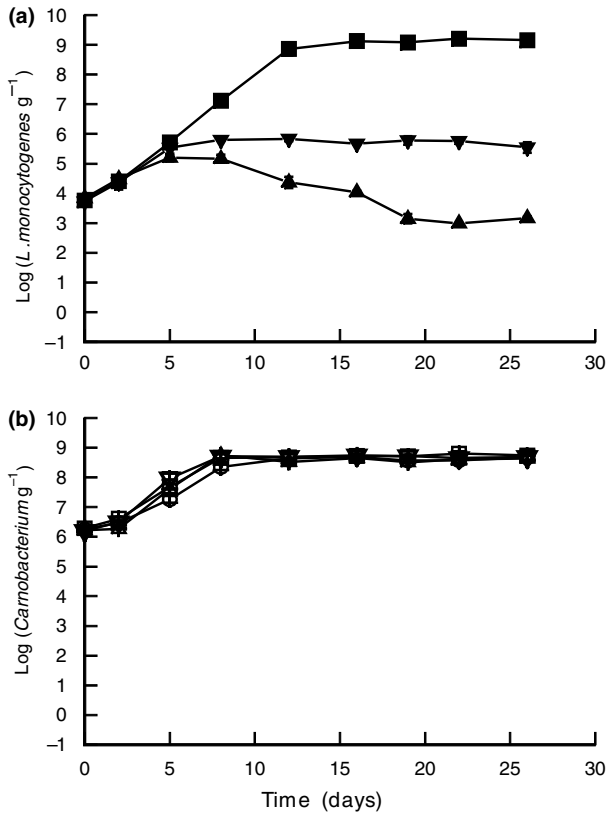

Fig. 3 Growth of *Listeria monocytogenes* O57 (a) and *Carnobacterium piscicola* (b) as monocultures and co-cultures in buffered BHI broth with 4% NaCl at 5°C. *L. monocytogenes* O57 alone (■); *L. monocytogenes* as a co-culture with *C. piscicola* A9b bac$^+$ (▲); *L. monocytogenes* as co-culture with *C. piscicola* A9b bac$^-$ (▼); *C. piscicola* A9b bac$^+$ with *L. monocytogenes* (△); *C. piscicola* A9b bac$^-$ with *L. monocytogenes* (▽); *C. piscicola* A9b bac$^+$ alone (○); *C. piscicola* A9b bac$^-$ alone (□). The error bars indicate mean and standard deviations of duplicate determinations

In the mixed-culture experiments, the inoculum ratios of *C. piscicola* A9b bac$^+$ or bac$^-$ with *L. monocytogenes* were $10^3 : 10^3$, $10^6 : 10^3$ and $10^3 : 10^6$ CFU ml^{-1} (Table 2). Inoculum size had a large effect on the growth of *L. monocytogenes* in mixed cultures with *C. piscicola* A9b bac$^+$ and bac$^-$. A low inoculum level of *L. monocytogenes* (10^3 CFU ml^{-1}) was inhibited in mixed culture with a low (10^3 CFU ml^{-1}) and a high (10^6 CFU ml^{-1}) inoculum level of *C. piscicola* A9b bac$^-$ which caused a 3 and 5 log reduction in maximum cell density of *L. monocytogenes*, respectively, compared with the similar monoculture of *L. monocytogenes*. The cell density of *L. monocytogenes* at the end of storage was reduced 3 and 7 log units in co-cultures with a low (10^3 CFU ml^{-1}) and a high (10^6 CFU ml^{-1}) inoculum level of *C. piscicola* A9b bac$^+$, respectively. Carnobacteriocin B2 was detected after 7 and 14 days of incubation in the presence of a low and high inoculum level of *C. piscicola* A9b

Table 2 Growth of *Listeria monocytogenes*, *Carnobacterium piscicola* A9b bac$^+$ and bac$^-$ as mono- or co-cultures in B-BHI broth at 5°C

	Log (CFU ml^{-1}); intial			Log (CFU ml^{-1}) after 28 days of storage		
Culture	*L.mono.*	*C.p.* bac$^+$	*C.p.* bac$^-$	*L.mono.*	*C.p.* bac$^+$	*C.p.* bac$^-$
Monoculture	3 or 6			9		
		3 or 6			9	
			3 or 6			9
Co-cultures	3	3		6	9	
	3	6		2	9	
	6	3		9	8	
	3		3	6		9
	3		6	4		9
	6		3	9		8

bac$^+$, respectively (data not shown). A low inoculum level of *L. monocytogenes* did not influence the growth of *C. piscicola* A9b (Table 2). The cell number of *C. piscicola* A9b (initial level of 10^3 CFU ml^{-1}) was reduced 1 log in the presence of a high inoculum size of *L. monocytogenes* (10^6 CFU ml^{-1}).

The antilisterial effect of *C. piscicola* A9b bac$^-$ coincided with the entry of A9b bac$^-$ into stationary phase for all inhibitory inocula (data not shown).

Inhibition of *L. monocytogenes* O57 in salmon juice and on cold-smoked salmon by *C. piscicola* A9b bac$^+$ and its bac$^-$ mutant

A sterile-filtered supernatant from *C. piscicola* bac$^+$ grown in cold-smoked salmon juice at 5°C inhibited growth of *L. monocytogenes* completely when the medium consisted of either 100 or 50% culture supernatant (Fig. 4). In contrast, *C. piscicola* bac$^-$ supernatant (100 or 50%) only caused a minor depression of maximum cell density. This depression was identical in size to the depression observed when an equal amount of sterile saline was added to the freshly prepared cold-smoked salmon juice (Fig. 4).

The antilisterial effect of wild-type *C. piscicola* A9b and its bac$^-$ mutant was tested in co-culture experiments in cold-smoked salmon juice at 5°C (Fig. 5a). *Carnobacterium piscicola* A9b bac$^+$ and bac$^-$ grew rapidly as pure cultures in salmon juice at 5°C, increasing from 2×10^6 CFU ml^{-1} to maximum counts of *ca* 10^8 CFU ml^{-1} after 6 days of incubation (Fig. 5b). The number of *L. monocytogenes* cells in pure culture increased from 10^4 to 5×10^8 CFU ml^{-1} after 15 days of storage. The growth of *L. monocytogenes* was strongly suppressed in mixed culture with *C. piscicola* A9b bac$^+$ and a 6 log reduction in the final listeria cell density was observed as compared with the control. The inhibition of *L. monocytogenes* coincided with the detection of carnobacteriocin B2. The initial level of 80 BU ml^{-1} did not change during storage. A bacteriostatic effect of *C. piscicola* A9b bac$^-$ could be observed after 7 days of incubation resulting in a 3 log reduction of the maximum cell number of *L. monocytogenes*.

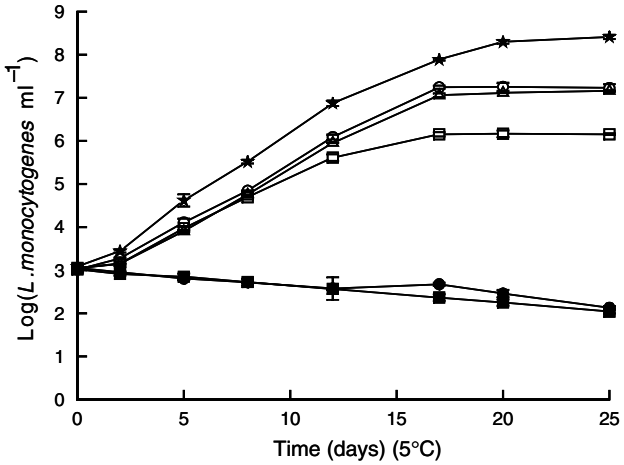

Fig. 4 Growth of *Listeria monocytogenes* O57 in sterile cold-smoked salmon juice (★); 100% (■) or 50% (●) culture supernatant from *Carnobacterium piscicola* bac$^+$; 100% (□) or 50% (○) culture supernatant from *C. piscicola* bac$^-$; and 50% sterile 4% saline (△). *C. piscicola* were cultured in cold-smoked salmon juice and all 50% cultures complemented with freshly prepared cold-smoked salmon juice. Curves are mean with errorbars indicating standard deviation of duplicate determinations

Exposure of listeria cells to precipitated carnobacteriocin (1024 BU ml^{-1}) from the wild-type strain caused a 3·5 log decline in viable cell count after 6 days (Fig. 5a). Regrowth of listeria cells was observed and the final level reached after 33 days of incubation was 10^3 CFU ml^{-1}. The resistance level of listeria cells during growth were measured by determining the MIC of carnobacteriocin B2. When cells were grown in the presence of an initial concentration of 1024 BU ml^{-1}, the MICs increased over time from 192 to >1536 BU ml^{-1} by day 6 for cells treated with purified

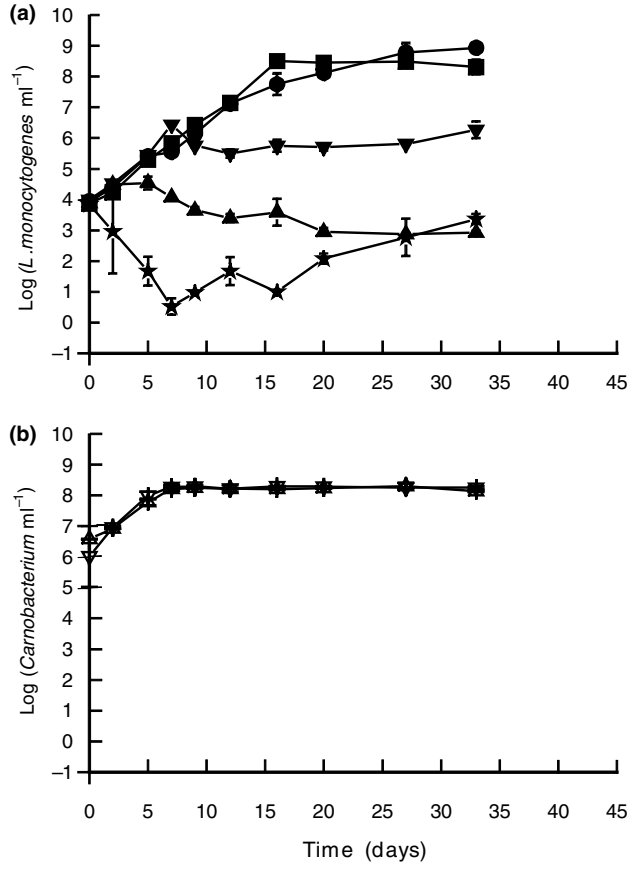

Fig. 5 Growth of *Listeria monocytogenes* O57 (a) and *Carnobacterium piscicola* (b) as monocultures and co-cultures in cold-smoked salmon juice with 4% NaCl at 5°C. *L. monocytogenes* O57 alone (■); *L. monocytogenes* as a co-culture with *C. piscicola* A9b bac$^+$ (▲); *L. monocytogenes* as co-culture with *C. piscicola* A9b bac$^-$ (▼); *L. monocytogenes* in the presence of precipitated supernatant of *C. piscicola* A9b bac$^+$ (★); *L. monocytogenes* in the presence of precipitated supernatant of *C. piscicola* A9b bac$^-$ (●); *C. piscicola* A9b bac$^+$ with *L. monocytogenes* (△); *C. piscicola* A9b bac$^-$ with *L. monocytogenes* (▽). The error bars indicate mean and standard deviations of duplicate determinations

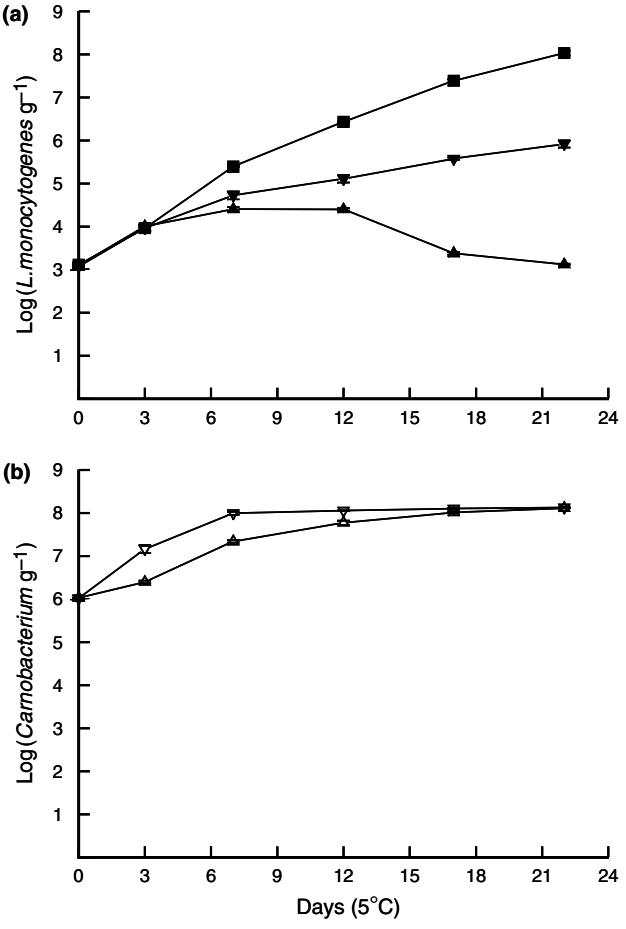

Fig. 6 Growth of *Listeria monocytogenes* O57 (a) and *Carnobacterium piscicola* (b) in vaccum-packed cold-smoked salmon stored at 5°C. *L. monocytogenes* as monoculture (■) or co-inoculated with *Carnobacterium piscicola* A9b bac$^+$ (▲) or *C. piscicola* A9b bac$^-$ (▼); *C. piscicola* A9b bac$^+$ with *L. monocytogenes* (△); *C. piscicola* A9b bac$^-$ with *L. monocytogenes* (▽). *C. piscicola* were inoculated at an initial level of 10^6 CFU g^{-1}. Errorbars indicate mean and standard deviations of duplicate determinations

carnobacteriocin (data not shown). For listeria cells grown in combination with living cells of *C. piscicola* A9b, the MICs increased from 192 to >1536 BU ml^{-1} by day 33. When listeria cells were grown as monoculture, the MIC was stable at 192 BU ml^{-1} during the storage time of 33 days.

Listeria monocytogenes grew well in vacuum-packed cold-smoked salmon at 5°C (Fig. 6). Co-inoculation with *C. piscicola* bac$^+$ resulted in reduction in maximum cell density from 10^8 to 10^4 CFU g^{-1}. The *C. piscicola* bac$^-$ caused a lesser reduction to 10^5–10^6 CFU g^{-1} (Fig. 6). As in laboratory media, the bac$^-$ variant grew slightly faster than the bacteriocin producing wild-type of *C. piscicola* strain A9b.

DISCUSSION

Previous work has shown that *C. piscicola* A9b isolated from cold-smoked salmon produced carnobacteriocin B2 which is an autoinducible, antilisterial class IIa bacteriocin (Nilsson *et al.* 2002). The specific role of bacteriocin production in the inhibition of *L. monocytogenes* was assessed in the present study by comparing the antilisterial effect of the wild-type parent strain with a bac$^-$ mutant of the wild-type strain. Our data suggest that the antilisterial effect of *C. piscicola* A9b in both laboratory medium and model fish systems depends on at least two mechanisms of action of which one, logically, is the production of carnobacteriocin B2. However, also the

nonbacteriocin-producing variant had a marked antilisterial effect.

Carnobacterium piscicola A9b bac$^-$ did not influence the growth rate of *L. monocytogenes* but suppressed growth after 5–8 days of incubation (at 5°C), which caused a reduction in final biomass yields in buffered BHI broth, in salmon juice and in cold-smoked salmon. The inhibition of *L. monocytogenes* on cold-smoked salmon by the bac$^-$ variant was, however, not as pronounced as by another nonbacteriocin-producing *Carnobacterium* used in other studies (Nilsson *et al.* 1999). Our data suggest that *L. monocytogenes* in mixed culture with *C. piscicola* A9b enter stationary phase earlier as compared with monocultures of *L. monocytogenes*. Buchanan and Bagi (1997) also showed that the effect of *C. piscicola* on *L. monocytogenes* was largely limited to a suppression of the maximum population density. One explanation for the behaviour of *L. monocytogenes* is exhaustion of one or a few essential nutrients and/or accumulation of toxic end products in the growth medium (Madigan *et al.* 1999; Finkel *et al.* 2000). The inhibitory effect of a sterile-filtered salmon juice from *C. piscicola* bac$^-$ was identical to that seen when sterile saline was added in the same volume. Further studies are required to determine the precise nature of the inhibitory mechanism. Competition for nutrients is considered to be one of the major evolutionary driving forces in the bacterial world (Christensen *et al.* 2002) and bacteria with a better utilization of a given energy source may outcompete other bacteria. Several studies have shown that the population of an indigenous microbial flora suppresses the growth of pathogens in foods (Mattila-Sandholm and Skytta 1991; Carlin *et al.* 1996; Babic *et al.* 1997; Samelis *et al.* 1998; Vold *et al.* 2000) probably due to a competitive advantage for nutrient uptake. It has also been suggested that a possible reason for the increasing number of food-borne outbreaks in the US is the low numbers of a competitive background flora to suppress pathogen survival and growth in foods (Jay 1996, 1997). Although microbial competition is an important safety issue in food manufacturing, only limited work have been conducted on the mechanisms of action that are not explained by the production of antimicrobial compounds. Such studies are needed, as an inhibitory effect of a bacteriocin-negative mutant cannot *a priori* be expected. Thus, Leisner *et al.* (1996) found that whilst a bacteriocin-producing *Leuconostoc* was strongly inhibitory towards a meat spoiling *Lactobacillus sake*, its bacteriocin-negative variant did not inhibit growth of the spoilage organism.

The extent of the *L. monocytogenes* suppression depended on the inoculum size of both *C. piscicola* A9b and *L. monocytogenes*. A low inoculum level of *L. monocytogenes* (10^3 CFU ml^{-1}) in mixed culture with a low (10^3 CFU ml^{-1}) and a high (10^6 CFU ml^{-1}) inoculum level of *C. piscicola* A9b bac$^-$ resulted in a 3 and 5 log reduction in maximum cell density of *L. monocytogenes*, respectively, compared with a monoculture of *L. monocytogenes*. Growth kinetics studies of bacterial pathogens in mixed cultures have also shown the significance of inoculum size. A uniform decrease in maximum cell density of the pathogenic bacterium with high levels of a competitive culture have been demonstrated (Drosinos and Board 1994; Buchanan and Bagi 1997, 1999; Breidt and Fleming 1998; Duffy *et al.* 1999; Vold *et al.* 2000). Bacteriocin production was initiated earlier in the presence of a high inoculum level of *C. piscicola* A9b bac$^+$ in B-BHI broth with 4% NaCl. We previously reported a similar effect of inoculum size on production of carnobacteriocin B2 in *C. piscicola* A9b, which was explained by a self-inducing cell density (quorum-sensing) mechanism (Nilsson *et al.* 2002). These data emphasizes the need to use a high initial level of *C. piscicola* A9b to improve the antilisterial efficiency.

Production of and immunity to carnobacteriocin B2 appeared to be plasmid associated, as loss of one or two plasmids of approx. 47 and 67 kb molecular size led to phenotypes of noncarnobacteriocin production and carnobacteriocin sensitivity. The phenotypic expression of carnobacteriocin B2 and the immunity functions conferring protection against the bacteriocin are also produced by a 61-kb plasmid from *C. piscicola* LV17B (Ahn and Stiles 1990; Quadri *et al.* 1994). *Carnobacterium piscicola* LV17B also produces the class IIa bacteriocin carnobacteriocin BM1 encoded on the chromosome (Quadri *et al.* 1994).

Cross-immunity to the class IIa bacteriocins bavaricin and leucocin was detected. However sensitivity to pediocin PA-1 and nisin was observed for both wild-type and the bac$^-$ phenotype. Comparative studies of class IIa bacteriocins have also shown that immunity proteins appear to provide partial protection against other class IIa bacteriocins (Eijsink *et al.* 1998; Ennahar *et al.* 2000). However exceptions were observed for the producers of sakacin P and curvacin A to be sensitive to the more potent pediocin PA-1 and enterocin A (Eijsink *et al.* 1998).

A comparative study between the parent strain and bac$^-$ mutant of *C. piscicola* A9b showed no phenotypic differences except for a higher growth rate of the bac$^-$ mutant, particularly at 5 and 37°C. Clearly, the plasmid does not encode any metabolic traits required for growth, and the faster growth rate of the mutant could be caused by its lower energy requirement for replication being noticeable at the slower growth rates.

The findings of the present study are of importance for the application of bioprotective cultures in the food industry as *C. piscicola* A9b can outcompete *L. monocytogenes* without production of bacteriocins. Much concern has been raised about the resistance of *Listeria* strains and

other pathogens to bacteriocins (Rekhif *et al.* 1994; Crandall and Montville 1998; Gravesen *et al.* 2002) and the use of nonbacteriocigenic cultures to overcome this obstacle. Further studies are needed to study competitive interactions of *L. monocytogenes* and *C. piscicola* in order to better understand microbial competition as a component of food safety systems and we are currently attempting to elucidate the exact mechanism of the antilisterial action by *C. piscicola* A9b bac$^-$.

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