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Two Subpopulations of *Listeria monocytogenes* Occur at Subinhibitory Concentrations of Leucocin 4010 and Nisin

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In situ analyses of single *Listeria monocytogenes* cells at subinhibitory concentrations of leucocin 4010 and nisin revealed two subpopulations when measured by fluorescence ratio imaging microscopy (FRIM) after staining with 5(6)-carboxyfluorescein diacetate succinimidyl ester. One subpopulation consisted of cells with a dissipated pH gradient (ΔpH), and the other consisted of cells that maintained ΔpH. The proportion of cells belonging to each subpopulation was estimated, and the concentrations of bacteriocins required to dissipate ΔpH for 90% of the cell population (ED_{90}) was predicted. ED_{90} increased after the addition of sodium chloride (1 to 3% [wt/vol]) to the bacteriocin solutions, while ED_{90} decreased by the addition of sodium nitrite (60 and 100 ppm). Other meat additives, including sodium phosphate, sodium lactate, sodium citrate, and sodium acetate slightly increased ED_{90}. The inhibitory effect of sodium chloride on the antilisterial activity of leucocin 4010 and nisin was confirmed on the surfaces of meat sausages. This study highlights the important practical implications of applying subinhibitory concentrations of bacteriocins, which results in unaffected target cells. In situ analyses by FRIM in combination with modeling of single-cell data can be applied to ensure that sufficient concentrations of bacteriocins are used in food preservation.

The tolerance of *Listeria monocytogenes* to refrigeration temperatures, high concentrations of NaCl, and anaerobic conditions (5, 14, 19) may necessitate the use of additional preservation for various food products such as vacuum-packed ready-to-eat meat products. The use of protective cultures, which produce bacteriocins or exert other kinds of competitive exclusion, may satisfy the demand for safe, fresh, and more natural meat products as proposed by many researchers (6, 11, 17).

Successful applications of bacteriocins and bacteriocin-producing strains have been demonstrated in various meat products (1, 11, 18, 31). However, in some cases survival of *L. monocytogenes* has been observed in foods after exposure to bacteriocins (1, 18, 31). Several studies have evaluated the activity of different bacteriocins, and they concluded that the bacteriocin concentration is critical to achieve sufficient inhibitory effect (1, 2, 24). An immediate reduction of *L. monocytogenes* that was inoculated onto vacuum-packaged meat sausages was only obtained when high concentrations of *Leuconostoc carnosum* 4010 (6.3×10^6 CFU/g) was applied (11). In addition to high bacteriocin concentrations, an even distribution of the bacteriocin-producing strain on a meat surface (27), and close contact between the protective culture and target organism have recently been identified as critical parameters for obtaining adequate competitive exclusion of *L. monocytogenes* (24). Survival of *L. monocytogenes* in meat products may also be prevented by additional use of various meat additives. Thus, to eliminate survivors and growth of *L. monocytogenes* in meat products, the combined effect of bacteriocins and additives still needs to be examined (24, 28, 33, 34).

Even though it has been emphasized that adequate concentrations of bacteriocins are important to obtain sufficient inhibition of *L. monocytogenes*, no detailed reports on the effect of bacteriocins at subinhibitory concentrations seem to be available. It is currently unknown whether treatment with subinhibitory concentrations of bacteriocin slightly affects the entire cell population or only attacks a fraction of the cell population, leaving another fraction of cells unaffected. Previously, we have performed in situ analyses of the interaction between bacteriocins and single cells of *L. monocytogenes* on a solid surface with the use of fluorescence ratio imaging microscopy (FRIM) (9). FRIM was used to determine the dissipation of the pH gradient (ΔpH) after exposure to nisin, and this setup revealed single cells of *L. monocytogenes* that maintained ΔpH depending on the history of the cells (9). Furthermore, the potential of this method to measure the interaction between bacteriocins and *L. monocytogenes* on the surfaces of food was highlighted (9).

The aim of the present study was to investigate the efficacy of leucocin 4010 or nisin at subinhibitory concentrations on single cells of *L. monocytogenes*. We applied the in situ technique FRIM on a solid surface to determine the heterogeneity with regard to pH_i of cells within a population of *L. monocytogenes*. Furthermore, we examined the antilisterial activity of the bacteriocins in combination with various meat additives and verified these results directly on surfaces of meat sausages. Modeling of bimodal data was used to predict the concentration of bacteriocin needed to dissipate ΔpH for 90% of the cells when applied in combination with various meat additives.

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MATERIALS AND METHODS

Chemicals. The chemicals used were of analytical grade and supplied by Merck (Darmstadt, Germany) unless otherwise stated.

Bacterial strains and growth conditions. *Leuconostoc carnosum* 4010 was isolated from vacuum-packed sliced ham as previously described (11) and is now commercially available as B-SF-43 from Chr. Hansen A/S, Hørsholm, Denmark. It was routinely grown for 48 h at 20°C in brain heart infusion (BHI; Difco, Detroit, Michigan) adjusted to pH 6.0 by using 1 M HCl. *Listeria monocytogenes* 4140 (isolated from bacon) was kindly provided by the Danish Meat Research Institute (Roskilde, Denmark). *L. monocytogenes* 4140 was routinely grown for 18 h at 37°C in BHI (pH 6.0). The strains were maintained in 20% (vol/vol) glycerol as frozen stock cultures at −80°C.

Bacteriocins. Production of leucocin 4010 was carried out by using the method described by Budde et al. (11). Briefly described, *Leuconostoc carnosum* 4010 was grown in acetate-free MRS (26) containing 5% (wt/vol) glucose adjusted to pH 6.5 at 20°C for 48 h using agitation (50 rpm). Catalase (0.2 g/liter; Sigma, Montana) was added to the fermentate, and cells were removed by centrifugation at 16,300 × *g* for 10 min. Leucocin 4010 was partially purified by ammonium sulfate precipitation (40% [wt/vol]) and dialysis (Spectra/Por Dialysis Membrane; Spectrum Laboratories, Inc., California) with a 1-kDa cutoff. The partially purified leucocin 4010 was filter sterilized (0.20 μm; Minisart, Sartorius AG, Göttingen, Germany) and kept at −80°C until use. Bacteriocin activity, expressed in arbitrary units (AU) per milliliter, was determined by the microtiter assay system as described by Budde and Rasch (10) with *L. monocytogenes* 4140 as an indicator strain. Nisin (Applin & Barrett, Ltd., Danisco-Cultor, Beaminster, Dorset, England) was prepared as a stock solution in 0.05 M sodium phosphate buffer (pH 6.0) containing 10 mM glucose.

Fluorescence labeling of cells and immobilization. Staining of *L. monocytogenes* 4140 with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Inc., Eugene, Oreg.) was carried out by using the method of Budde and Jakobsen (9). Cells were harvested by centrifugation at 10,400 × *g* for 5 min and resuspended in sterile-filtered (pore size, 0.22 μm; GP Express Membrane Filter; Millipore, Bedford, Mass.) phosphate-buffered saline (pH 7.4) containing 0.15% (wt/vol) Na_2HPO_4, 0.022% (wt/vol) NaH_2PO_4, and 0.85% (wt/vol) NaCl. The fluorochrome, CFDA-SE, was added to obtain a final concentration of 10 μM, and the cell suspension was incubated at 37°C for 30 min. Cells were harvested by centrifugation at 10,400 × *g* for 5 min and resuspended in 50 mM sterile-filtered potassium phosphate buffer (pH 6.0) supplemented with glucose (10 mM). The cell suspension was energized at 30°C for 30 min, harvested by centrifugation at 10,400 × *g* for 5 min, and resuspended in 50 mM sterile-filtered potassium phosphate buffer (pH 6.0) supplemented with glucose (10 mM). Stained cells were kept on ice until analysis and no longer than 2 h. Stained cells of *L. monocytogenes* 4140 were immobilized on a membrane filter (ME 25/31; Schleicher & Schuell, Dassel, Germany) by using the method described by Budde and Jakobsen (9) or on a slice of sausage before microscopic examinations.

FRIM. FRIM measurements of *L. monocytogenes* 4140 were carried out using the setup described by Guldfeldt and Arneborg (21). This setup consisted of an inverted epifluorescence microscope (Axiovert 135 TV; Zeiss, Birkerød, Denmark) equipped with a Zeiss Fluar ×100 objective (numerical aperture, 1.3), a dichroic mirror (510 nm), and an emission band-pass filter (515 to 565 nm). Cells were excited at 490 and 435 nm with exposure times of 3 s by a monochromator equipped with a 75-W xenon lamp (Monochromator B; TILL Photonics GmbH, Planegg, Germany). To minimize photo bleaching of the stained cells, a 2.5% neutral-density filter was inserted between the optical fiber and the microscope. Fluorescence emission was collected with a cooled charge-coupled device camera (EEV 512 × 1024, 12-bit frame camera; Princeton Instruments) and images were analyzed by using the Metafluor 4.5 software (Universal Imaging Corp., West Chester, Pa.). Regions were defined around approximately 100 individual cells for each image by using Metafluor, and for each region ratio values ($R_{490/435}$) were obtained by dividing the fluorescence intensity at 490 nm (pH sensitive wavelength) with the fluorescence intensity at 435 nm (a pH-insensitive wavelength).

Exposure of *L. monocytogenes* 4140 to leucocin 4010 and nisin in combination with meat additives. Membrane filters or slices of sausages with the immobilized cells (ca. 10^6 and 10^7, respectively) were placed in a chamber containing a 300-μl solution of leucocin 4010 or nisin in combination with meat additives, including sodium chloride (1, 2, and 3% [wt/vol]), sodium nitrite (60 and 100 ppm), sodium citrate (0.2 and 0.4% [wt/vol]), sodium phosphate (0.155 and 0.31% [wt/vol]), sodium lactate (0.5 and 1.0% [wt/vol]), or sodium acetate (0.125 and 0.35% [wt/vol]). Experiments on membrane filters were carried out at pH 6.0. Sausages were prepared according to the method of Budde et al. (11) with the addition of selected meat additives including sodium chloride (2% [wt/vol]), sodium nitrite (60 ppm), sodium citrate (0.4% [wt/vol]), sodium lactate (1% [wt/vol]), or sodium acetate (0.25% [wt/vol]), and the pH of the sausages was ca. 6.3. For each combination of bacteriocin and meat additive, microscopic images were acquired in triplicates on each sample with duplicate experiments. Images were acquired within 5 min after exposure of the cells to the bacteriocin solutions.

Determination of the number of viable cells of *L. monocytogenes* after exposure to bacteriocins. To determine the number of viable *L. monocytogenes* cells after exposure to bacteriocins for 5 min, the membrane filters were aseptically removed from the microscope chamber and transferred to 10 ml of peptone saline solution. The membrane filters were thoroughly vortexed, and tenfold dilutions made in peptone saline solution were plated on BHI agar (Oxoid, Ltd., Basingstoke, United Kingdom). CFU were enumerated after incubation for 48 h at 37°C.

Statistical methods. A novel statistical method was developed to describe the dissipation of ΔpH for single cells of *L. monocytogenes* exposed to the bacteriocins. Statistical analyses were carried out by using $R_{490/435}$ values. In all experiments, single-cell analysis of $R_{490/435}$ values revealed a bimodal distribution (i.e., two subpopulations) as outlined in Fig. 2: one subpopulation showed an average $R_{490/435}$ of ca. 2.5 (subpopulation 1), and the other subpopulation showed an average $R_{490/435}$ of ca. 9 (subpopulation 2). For each of the different combinations of bacteriocin and meat additive, the following order of procedures was used (each step of the statistical procedure is explained in details in the Appendix). For step 1, the proportion of cells belonging to each subpopulation was estimated by fitting a normal mixture model to the ratio values of individual cells for each image. For step 2, the relation between bacteriocin concentration and the proportion of cells in each subpopulation was estimated by weighted logistic regression. For step 3, predictions of the concentration of bacteriocin corresponding to 90% of the cells belonging to subpopulation 1 (ED_{90} value), as well as the approximate standard errors, were made.

RESULTS

Ratio images of *L. monocytogenes* upon exposure to various concentrations of leucocins 4010 or nisin are seen in Fig. 1. In Fig. 1A and D, ratio images for cells not being exposed to bacteriocins are shown, and the $R_{490/435}$ of *L. monocytogenes* was approximately 9. This value corresponded to pH_i 7.9 according to Budde and Jakobsen (9), and at the extracellular pH (pH_{ex}) of 6.0, cells maintained a ΔpH of approximately 2 pH units. Ratio images of cells exposed to high concentrations of leucocins (24,000 AU/ml) and nisin (1800 IU/ml) are shown in Fig. 1C and F, and the $R_{490/435}$ of cells was approximately 2.5, corresponding to pH_i 6.0 according to Budde and Jakobsen (9). At subinhibitory concentrations of leucocin 4010 (9,600 AU/ml) and nisin (400 IU/ml), ΔpH dissipated in a fraction of the cells, while the remaining part of the cells maintained their ΔpH within the time of exposure (5 min) which is also reflected in Fig. 1B and E. The viable count of *L. monocytogenes* 4140 decreased from 5.5×10^5 to 2.3×10^5 CFU/ml after treatment with 9,600 AU of leucocin 4010/ml and from 2.5×10^5 to 6×10^4 CFU/ml after treatment with 400 IU of nisin/ml. Exposure to higher concentrations of leucocin (24,000 AU/ml) and nisin (1,800 IU/ml) caused ΔpH dissipation in all *L. monocytogenes* cells examined as seen in Fig. 1C and F. However, the viable count only decreased from 5.5×10^5 to 2.3×10^5 CFU/ml after exposure to 24,000 AU of leucocin 4010/ml, whereas the viable count decreased from 2.5×10^5 to 1.4×10^2 CFU/ml after exposure to 1,800 IU of nisin/ml.

More detailed information on the distributions of the measured $R_{490/435}$ values shown as bars, as well as the fitted curves of the binomial distributions of the $R_{490/435}$ values (estimated according to steps i and ii of the statistical procedure), are shown in Fig. 2 and 3 for *L. monocytogenes* exposed to leucocin 4010 and nisin, respectively. In the absence of bacteriocins, all

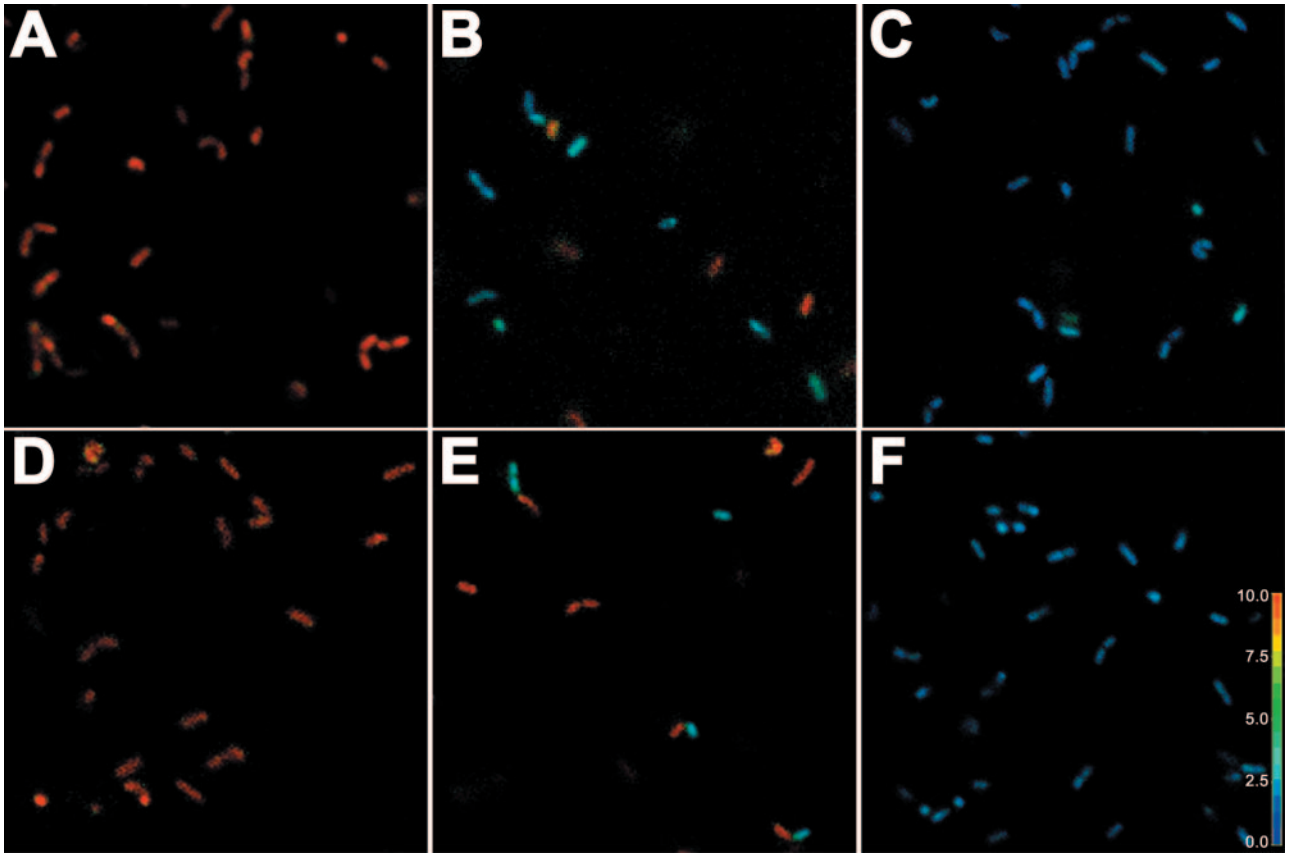

FIG. 1. Ratio images of *L. monocytogenes* 4140 cells immobilized on a filter membrane at an extracellular pH of 6.0 and exposed to leucocin at concentrations of 0 (A), 9,600 (B), and 24,000 (C) AU/ml or nisin at concentrations of 0 (D), 400 (E), and 1,800 (F) IU/ml. A color-coded $R_{490/435}$ scale is shown on the right.

L. monocytogenes cells were designated as subpopulation 2, showing $R_{490/435}$ values with an average of ca. 9.0 (Fig. 2a and 3a). Exposure of *L. monocytogenes* to subinhibitory concentrations of leucocin 4010 (9,600 AU/ml) or nisin (400 IU/ml) revealed two subpopulations (Fig. 2b and 3b). One subpopulation showed low values of $R_{490/435}$, with an average of ca. 2.5, and these cells were designated as subpopulation 1, and the other cells with high values of $R_{490/435}$ (average of ca. 9.0) were designated as subpopulation 2 (Fig. 2b and 3b). However, the histograms in Fig. 2b and 3b also show that a minor fraction of cells exhibited $R_{490/435}$ values in between the typical low value (μ_{low}) and the typical high value (μ_{high}) calculated as explained in step i of the statistical procedure. These cells were assigned to subpopulation 1 or 2 according to the weighting scheme described in step ii of the statistical analysis. After exposure to sufficiently high concentrations of leucocin 4010 (24,000 AU/ml) and nisin (1,800 IU/ml), all *L. monocytogenes* cells examined were designated as subpopulation 1 (Fig. 2c and 3c).

Within the time of exposure, meat additives alone did not affect the number of cells designated to subpopulation 2 but slightly changed the value of μ_{high} for this subpopulation (results not shown). Upon exposure to leucocin 4010 in combination with sodium chloride, the percentages of cells designated to the two subpopulations were estimated by using weighted logistic regression. Addition of sodium chloride decreased the antilisterial effect of leucocin 4010, and it was necessary to increase the concentrations of leucocin 4010 to obtain the same effect as without sodium chloride (Fig. 4). The concentration of bacteriocins required to dissipate ΔpH for 90% of the cells (ED_{90}) in the absence or in the presence of the various meat additives was predicted for each of the conditions (Table 1). ED_{90} increased from 1.9×10^4 to 1.1×10^6 AU/ml for leucocin 4010 when 3% (wt/vol) sodium chloride was added (Table 1). Sodium chloride also inhibited the activity of nisin, and ED_{90} increased from 4.1×10^2 to 1.1×10^4 IU/ml upon addition of 3% (wt/vol) sodium chloride (Table 1). Addition of either sodium phosphate (0.155 and 0.31% [wt/vol]), sodium lactate (0.125 and 0.25% [wt/vol]), sodium citrate (0.4% [wt/vol]), or sodium acetate (0.25% [wt/vol]) slightly decreased the antilisterial effect (Table 1). The addition of sodium nitrite (60 and 100 ppm) slightly enhanced the antilisterial effect in combination with either leucocin 4010 or nisin (Table 1). The addition of sodium nitrite (100 ppm) decreased the ED_{90} from 1.9×10^4 AU/ml to 1.7×10^4 AU/ml for leucocin 4010, whereas ED_{90} decreased from 420 to 310 IU/ml for nisin (Table 1).

The combined antilisterial effect of the bacteriocins and meat additives was verified by in situ measurements on the surface of meat sausages. A significant antilisterial effect of

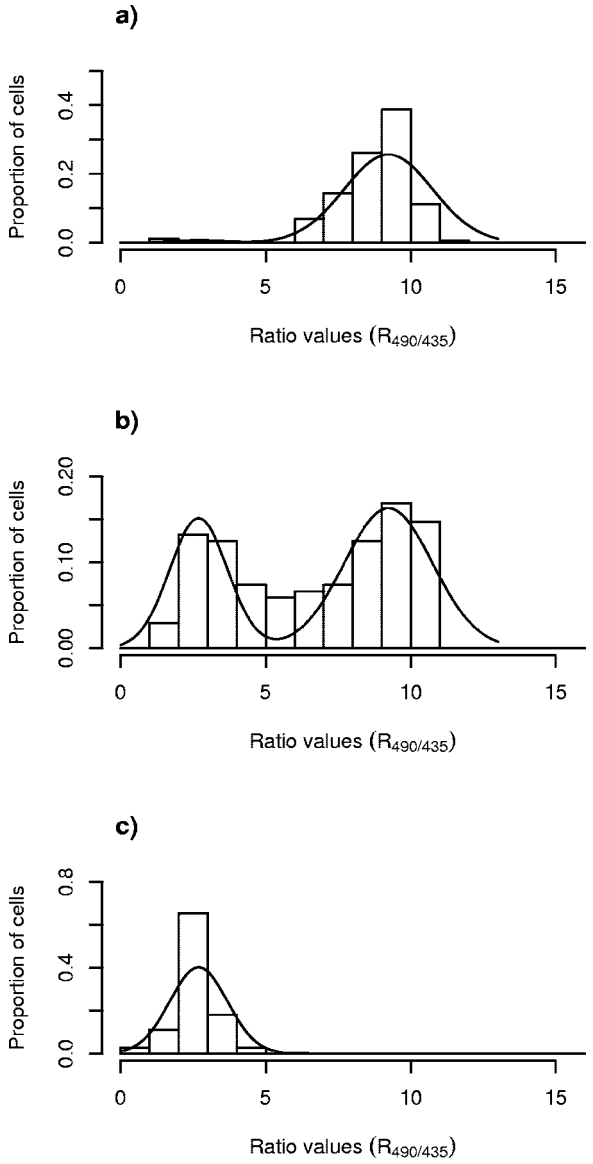

FIG. 2. Distributions of $R_{490/435}$ values of single cells of *L. monocytogenes* 4140 cells immobilized on a filter membrane and measured by FRIM upon exposure to leucocin 4010 at concentrations of 0 (a), 9,600 (b), and 24,000 (c) AU/ml.

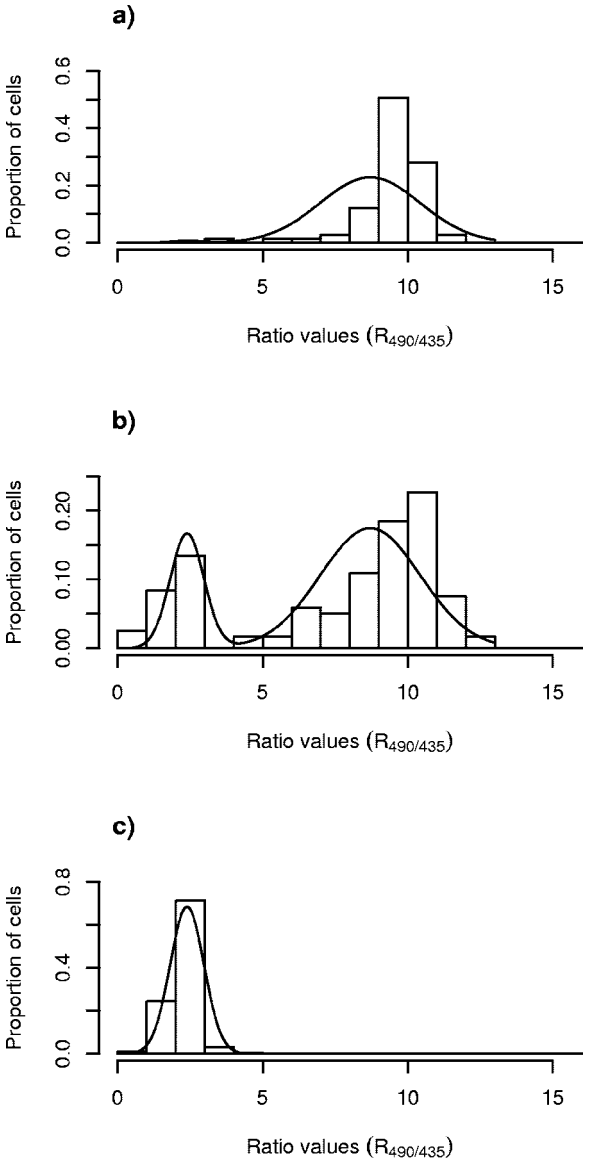

FIG. 3. Distributions of $R_{490/435}$ values of single cells of *L. monocytogenes* 4140 cells immobilized on a filter membrane and measured by FRIM upon exposure to nisin at concentrations of 0 (a), 400 (b), and 1,800 (c) IU/ml.

leucocin 4010 (48,000 AU/ml) was observed on surfaces of meat sausages without sodium chloride added, and ΔpH was dissipated for all cells (Fig. 5a). In contrast, when sodium chloride was added (2.5% [wt/vol]) to the meat sausage, the antilisterial effect of leucocin 4010 (48,000 AU/ml) decreased and at the end of the exposure time (5 min) less than half of the cells were designated to subpopulation 1, leaving more than half of the cells unaffected (Fig. 5b). Similar reductions in the antilisterial effect of nisin on meat surfaces were observed when sodium chloride was added to the meat matrix (results not shown). The smaller impact of the other meat additives on the antilisterial effect that was observed in the liquid system (Table 1) could not be verified when the additives were mixed into the meat matrix (results not shown).

DISCUSSION

Based on the two subpopulations behavior of *L. monocytogenes* observed in this study, it is likely that treatment of *L. monocytogenes* in meat products at subinhibitory concentrations of bacteriocins will result in the inactivation of a fraction of the population rather than weakening each individual cell of the population. This emphasizes the importance of using sufficient concentrations of bacteriocin in food products in order to eliminate survival and growth of surviving *L. monocytogenes*.

Bacteriocin molecules tend to aggregate and form large complexes due to their hydrophobic nature (7, 40). These properties potentially lead to localized gradients of bacteriocin molecules, which may be particularly critical at limited concen-

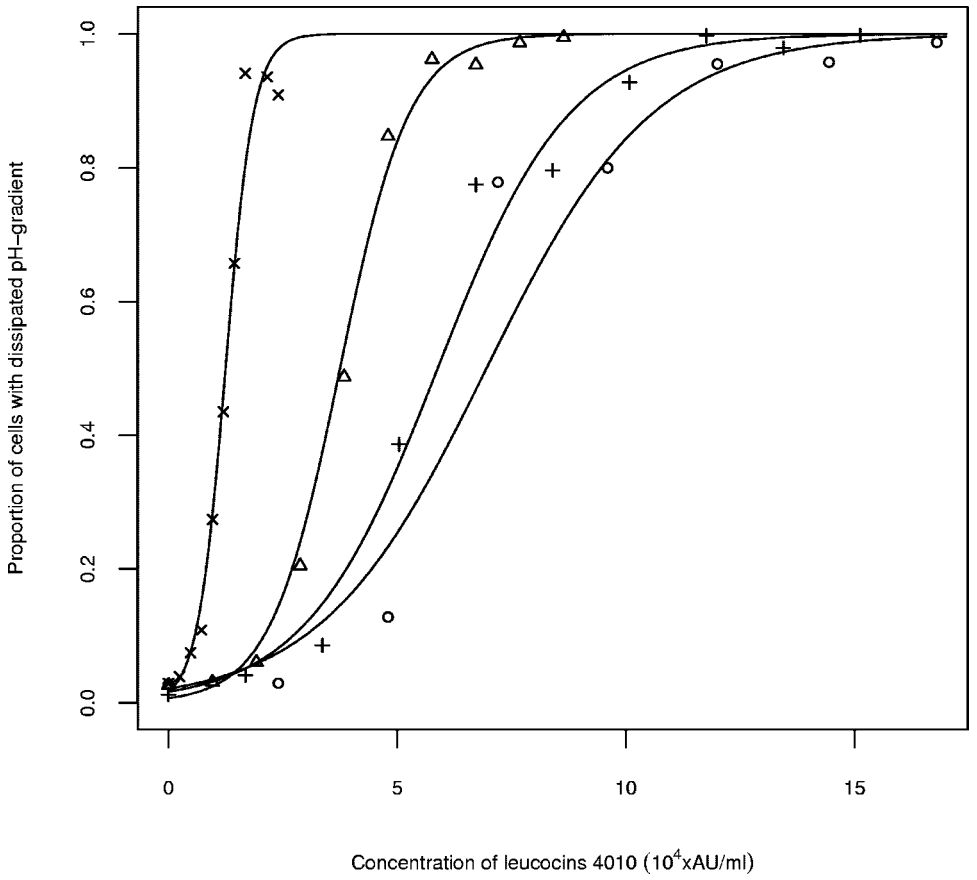

FIG. 4. Proportion of *L. monocytogenes* 4140 cells assigned to subpopulation 1, with low $R_{490/435}$ values corresponding to cells with dissipated ΔpH after exposure to various concentrations of leucocin 4010 in combination with sodium chloride at percent (wt/vol) concentrations of 0 (×), 1 (△), 2 (+), and 3 (○).

trations of bacteriocin. The presence of localized gradients is the most reasonable explanation for the two subpopulations observed in the current study. Bimodal distributions may be even more pronounced in solid foods such as meat products containing bacteriocin-producing strains, since the transmission of bacteriocins happens through diffusion and not convection (30)

The occurrence of phenotypic heterogeneity of *L. monocytogenes* cannot be excluded as an explanation for the two subpopulations observed upon exposure to subinhibitory concentrations of bacteriocins. Phenotypic heterogeneity constitutes the nongenetic variation existing among individual cells within an isogenic population and is related to different levels of basal gene expression rather than different gene possession (39). Phenotypic heterogeneity arises via progression through the cell cycle (15) and is typically seen when cells are exposed to stressful conditions such as heat (3). Phenotypic heterogeneity has also been observed for *Escherichia coli* cells surviving and being persistent to antibiotic treatment (4). Furthermore, the heterogeneity of cells grown on solid substrates has been observed with respect to bacteriocin sensitivity, RNA content, and levels of carotenoid pigmentation (9, 13).

The bimodal distribution does probably not reflect genotypic heterogeneity within the *L. monocytogenes* population resulting in different susceptibilities of single cells to the bacteriocins. The frequency of genotypic heterogeneity of *L. monocytogenes* 412 (identical to strain 4140) concerning resistance to nisin and pediocin PA-1 has been thoroughly investigated and shown to be 10^{-4} and 10^{-6}, respectively (20). This low rate of bacteriocin resistance combined with the fact that ΔpH was dissipated for all single cells at high concentration of bacteriocin makes bacteriocin resistance an unlikely explanation to the bimodal distribution. Likewise, bacteriocin resistance did not seem to be the primary reason for *L. monocytogenes* surviving on sausages after exposure to leucocin 4010, since no resistant strains could be retrieved among the survivors (27). To confirm the suggested origin of the two subpopulations at subinhibitory concentrations of bacteriocin, detailed investigations of cells from each subpopulation should be performed in the future with the use of either fluorescence-activated cell sorting or micromanipulation.

Traditionally, the growth-inhibitory effect caused by bacteriocins added to foods has been determined by plate counting. Using this method, *L. monocytogenes* is transferred to optimal growth conditions, e.g., with regard to media not containing bacteriocin and to optimal temperature for growth. In the present study, exposure of *L. monocytogenes* to nisin resulted in a pronounced increase in the number of cells with dissipated ΔpH (subpopulation 1), which corresponded to a decrease in the viable count on BHI agar. However, exposure to leucocin

TABLE 1. ED_{90} values for *L. monocytogenes* 4140 upon exposure to leucocin 4010 or nisin and different meat additives

Meat additive (% [wt/vol])[a]	ED_{90}[b] (SD)	
	Leucocin 4010 (AU/ml, 10^4)	Nisin (IU/ml, 10^2)
No additive	1.9 (0.6)	4.2 (0.2)
Sodium chloride		
1.0	54.1* (1.1)	38* (1.1)
2.0	90.5* (2.6)	71.2* (2.1)
3.0	109.4* (4.1)	108.3* (4.6)
Sodium phosphate		
0.155	2.7* (0.8)	5.6* (0.2)
0.31	3.7* (0.7)	8.5* (0.3)
Sodium acetate		
0.125	2.0 (0.7)	5.2* (0.2)
0.25	2.3* (0.9)	6.8* (0.2)
Sodium lactate		
0.5	2.2* (0.5)	6.1* (0.2)
1.0	2.8* (1.0)	8.2* (0.4)
Sodium citrate		
0.2	2.6* (0.5)	4.2 (0.2)
0.4	3.1* (0.6)	5.1* (0.2)
Sodium nitrite		
60 ppm	1.6† (0.8)	3.4† (0.1)
100 ppm	1.7† (0.7)	3.1† (0.1)

[a] Values are percentages except as noted.

[b] The value is an estimated average. *, the ED_{90} value is significantly ($P < 0.05$) higher than the ED_{90} value observed in the absence of meat additives; †, the ED_{90} values is significantly ($P < 0.05$) lower than the ED_{90} value observed in the absence of meat additives.

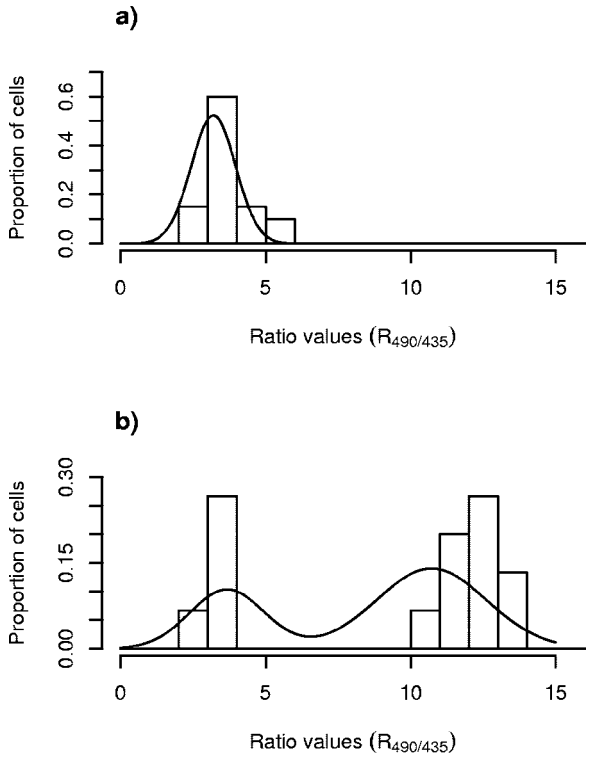

FIG. 5. Distributions of $R_{490/435}$ values of single cells of *L. monocytogenes* 4140 cells immobilized on slices of sausages containing no sodium chloride (a) or 2.5% (wt/vol) sodium chloride (b) after exposure to leucocin 4010 at a concentration of 48,000 AU/ml.

4010 at concentrations that dissipated ΔpH for all cells only resulted in ca. 50% reduction in the viable count, which may be explained by recovery of *L. monocytogenes* upon transfer to BHI agar. The existence of such a recovery mechanism was supported by the observation of very different colony sizes, including pinpoint colonies of *L. monocytogenes* after exposure to leucocin 4010 (results not shown). A repair mechanism was also demonstrated in *Lactobacillus* spp. after bacteriocin treatment, when viable counts were compared to direct observation of pore formation by flow cytometry (10). Similar recovery was not observed on a meat sausage (11, 27), which may be related to the concurrent presence of leucocin 4010 and *Listeria monocytogenes* and potentially the combined effect of a hostile sausage environment. These observations highlight the importance of in situ investigations of bacteriocins and their target organisms, as demonstrated in the present study. Previously, FRIM has been used to measure pH_i in *L. monocytogenes* (9, 37), *Bacillus licheniformis* (23), *Candida krusei*, and *Saccharomyces cerevisiae* (22), as well as lactic acid bacteria (38), on solid surfaces such as membrane filters or poly-L-lysine-coated glass slides. In the present study, FRIM was successfully applied for measuring pH_i directly on the surface of meat sausages, and in the future these in situ measurements may advantageously be performed on other food surfaces.

Within the short time of exposure (5 min), meat additives alone showed no effect on the proportion of *L. monocytogenes* cells designated to the two subpopulations (results not shown). Sodium chloride inhibited the activity of leucocin 4010 and nisin demonstrating that a higher amount of bacteriocin was required to inactivate all *L. monocytogenes* cells on meat sausages containing 3% (wt/vol) NaCl compared to products containing lower concentrations of NaCl. The inhibitory effect of NaCl on leucocin 4010 and nisin is in accordance with the inhibitory effect of NaCl on the antilisterial effect of pediocin, and this inhibition was negated by increasing the concentration of bacteriocin (8). Similar effects of sodium chloride was observed on the growth-inhibitory effect of *Leuconostoc carnosum* 4010 (24), sakacin K (25), lactocin (41), and nisin and pediocin (16, 28). Decreased activities of various bacteriocins in the presence of sodium chloride have been explained by chloride anions inhibiting the binding of bacteriocin to the cell surface of a target organism (8), sodium chloride inducing conformational changes of bacteriocins (29), or changing the envelope of the target organism (28).

A decrease in the activities of leucocin 4010 and nisin was observed in the presence of sodium phosphate, sodium acetate, sodium lactate, and sodium citrate (Table 1). Previously, the addition of phosphate at concentrations of 40 mmol/liter or higher was shown to inhibit the effect of nisin, and this effect was ascribed to the ability of the phosphate ions to block the electrostatic interaction between bacteriocin and cell membrane (8). It is not known whether the decrease in bacteriocin activities when applied in mixtures with the above-mentioned meat additives could be explained by a similar ion-blocking effect. In contrast, previous work has demonstrated a synergis-

tic growth-inhibitory effect of sodium lactate (1.8 to 3.6% [wt/vol]) and nisin (4,000 to 6,000 IU/ml) against *L. monocytogenes* (33) and a synergistic growth-inhibitory effect of sodium lactate (2% [wt/vol]) or sodium citrate (1.5% [wt/vol]) in combination with nisin (500 IU/ml) against *Arcobacter butzleri* (35). The different results may be explained by the different times of exposure to bacteriocins and meat additives for the target organism since only the initial activity of bacteriocins was determined by FRIM and not a possible long-term effect of the meat additives on the bacteriocin activity. Increased antilisterial activity was also demonstrated for *Leuconostoc carnosum* 4010 immobilized in a structured gelatin system after addition of sodium nitrite (60 or 100 ppm) (24). In contrast, sodium nitrite decreased the growth-inhibitory effect of lactocin 705 against *L. monocytogenes* (41).

In conclusion, we show here that the use of subinhibitory concentrations of leucocin 4010 and nisin resulted in two subpopulations of *L. monocytogenes* where only one subpopulation was affected by the treatment rather than weakening each individual cell of the population. The apparent recovery of *L. monocytogenes* cells when removed from leucocin 4010 to optimal growth conditions on BHI agar plates for estimation of viable counts emphasizes the importance of using in situ measurements to examine the effect of bacteriocins. In situ measurements of the activity of leucocin 4010 or nisin in combination with various meat additives were successfully performed on surfaces of meat sausages. Sodium chloride decreased the activity of leucocin 4010 and nisin when applied in a solution, as well as in a meat matrix. The effectiveness of bacteriocins should carefully be evaluated in food products containing the desired levels of additives since these may affect the concentrations of bacteriocins needed to produce safe food products.

APPENDIX

Step 1. The distribution of $R_{490/435}$ values in an image Y_{ij} was described by a bimodal distribution with a mode ("top") around the typical values μ and the dispersion σ of $R_{490/435}$ values for cells belonging to subpopulation 1 and 2, respectively: $Y_{ij} \approx N(\mu_{low}, \sigma_{low}^2)$, if cell j in image i had a low $R_{490/435}$ value (subpopulation 1); and $Y_{ij} \approx N(\mu_{high}, \sigma_{high}^2)$, if cell j in image i had a high $R_{490/435}$ value (subpopulation 2)

The (unobserved) status of each individual cell was modeled by binomial distributions bin(1, p_i) with the underlying proportion p_i depending on the image. The unknown true p_i was the key figure that expressed the proportion of cells in subpopulation 1. The model is a well-defined statistical model, known as a normal mixture model, in which estimates of the unknown model parameters could be found by a maximum-likelihood estimation, often carried out by the EM algorithm (32). For a single image this could be accomplished by commercial statistical software with standard clustering routines, but for several images simultaneously it was not possible and the applied algorithm is given explicitly in the following:

(i) Starting values for p_i, μ_{low}, σ_{low}, μ_{high}, and σ_{high} were chosen.

(ii) E-step. The probability of belonging to subpopulation 1 α_{ij} was calculated for each cell.

$$\alpha_{ij} = \frac{p_i \varphi\left(\frac{y_{ij} - \mu_{low}}{\sigma_{low}}\right) \Big/ \sigma_{low}}{(1 - p_i)\varphi\left(\frac{y_{ij} - \mu_{high}}{\sigma_{high}}\right) \Big/ \sigma_{high} + p_i \varphi\left(\frac{y_{ij} - \mu_{low}}{\sigma_{low}}\right) \Big/ \sigma_{low}} \tag{1}$$

where φ was the standard normal density function.

(iii) M-step. The proportion of cells belonging to subpopulation 1 was estimated by calculating the average of the individual cell probabilities.

$$p_i = \frac{\sum_j \alpha_{ij}}{n_i} \tag{2}$$

Estimates of the two intensity value distributions were calculated by using the individual cell probabilities as weights as follows:

$$\mu_{low} = \frac{\sum_{ij} \alpha_{ij} y_{ij}}{\sum_{ij} \alpha_{ij}}, \sigma_{low}^2 = \frac{\sum_{ij} \alpha_{ij} (y_{ij} - \mu_{low})^2}{\sum_{ij} \alpha_{ij}}$$

$$\mu_{high} = \frac{\sum_{ij} (1 - \alpha_{ij}) y_{ij}}{\sum_{ij} (1 - \alpha_{ij})}, \sigma_{high}^2 = \frac{\sum_{ij} (1 - \alpha_{ij}) (y_{ij} - \mu_{high})^2}{\sum_{ij} (1 - \alpha_{ij})} \tag{3}$$

(iv) Iteration was carried out between steps ii and iii until convergence was obtained.

Step 2. Using a standard logistic regression procedure, the SAS Genmod Procedure (36), the relation between the p_i values deduced from step 1 and the corresponding bacteriocin concentration C_i was modeled by a logistic curve:

$$\log\left(\frac{p_i}{1 - p_i}\right) = \alpha + \beta C_i \tag{4}$$

This analysis required binomial data for each observation i, i.e., a number of "observed successes" and the number of total possible successes, in this case, the expected number of cells belonging to subpopulation 1 corresponding to $n_i p_i$ out of n_i possible. Weights, w_i, which were used to determine the probability of a cell to belong to each of the subpopulations, were calculated according to the weighting scheme given by:

$$w_i = \frac{p_i(1 - p_i)}{n_i} \cdot \sum_{j=1}^{n_i} \frac{\left[\varphi\left(\frac{y_{ij} - \mu_{high}}{\sigma_{high}}\right) \Big/ \sigma_{high} - \varphi\left(\frac{y_{ij} - \mu_{low}}{\sigma_{low}}\right) \Big/ \sigma_{low}\right]^2}{\left[(1 - p_i)\varphi\left(\frac{y_{ij} - \mu_{high}}{\sigma_{high}}\right) \Big/ \sigma_{high} + p_i \varphi\left(\frac{y_{ij} - \mu_{low}}{\sigma_{low}}\right) \Big/ \sigma_{low}\right]^2} \tag{5}$$

This choice of weights ensured that the uncertainty of the proportions p_i in the analysis equaled that given by using standard asymptotic statistics theory on the normal mixture model.

Approximate variances of the $R_{490/435}$ values in subpopulations 1 and 2 could be deduced by differentiating the log-likelihood function twice. In this case, the log-likelihood function differentiated twice with respect to p_i resulted in:

$$-\frac{n_i w_i}{p_i (1 - p_i)} \tag{6}$$

To account for variations in the p_i values that were likely to occur due to sampling, replication, and lack of logistic curve fit, the weighted logistic regression was carried out with a so-called overdispersion factor. The deviance scale option of the procedure was chosen (36).

Step 3. The concentration level corresponding to 90% cells belonging to subpopulation 1 with low $R_{490/435}$ ED_{90} values was derived from the logistic relation:

$$ED_{90} = \frac{\log(0.90/0.10) - \alpha}{\beta} \tag{7}$$

Since this was a function of the two logistic curve parameters, the approximate uncertainty of the ED_{90}, SE_{ED90}, was deduced by standard error analysis (12), giving:

$$SE_{ED_{90}} = \sqrt{\frac{1}{\beta^2}\left(s_\alpha^2 + \frac{(\log(9)-\alpha)^2}{\beta^2} s_\beta^2 - 2\frac{(\log(9)-\alpha)}{\beta} s_{\alpha\beta}\right)} \quad (8)$$

where s_α^2 and s_β^2 were the estimation variances of α and β and $s_{\alpha\beta}$ was the estimation covariance, and all three values could be seen from standard logistic regression software output. Comparison of ED_{90} values for two different compounds was based on the uncertainty of a difference in ED_{90} values:

$$SE(ED_{90A} - ED_{90B}) = \sqrt{SE_{ED90A}^2 + SE_{ED90B}^2} \quad (9)$$

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