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Response of *Listeria monocytogenes* to Disinfection Stress at the Single-Cell and Population Levels as Monitored by Intracellular pH Measurements and Viable-Cell Counts[▽]

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***Listeria monocytogenes* has a remarkable ability to survive and persist in food production environments. The purpose of the present study was to determine if cells in a population of *L. monocytogenes* differ in sensitivity to disinfection agents as this could be a factor explaining persistence of the bacterium. In situ analyses of *Listeria monocytogenes* single cells were performed during exposure to different concentrations of the disinfectant Incimaxx DES to study a possible population subdivision. Bacterial survival was quantified with plate counting and disinfection stress at the single-cell level by measuring intracellular pH (pH_i) over time by fluorescence ratio imaging microscopy. pH_i values were initially 7 to 7.5 and decreased in both attached and planktonic *L. monocytogenes* cells during exposure to sublethal and lethal concentrations of Incimaxx DES. The response of the bacterial population was homogenous; hence, subpopulations were not detected. However, pregrowth with NaCl protected the planktonic bacterial cells during disinfection with Incimaxx (0.0015%) since pH_i was higher (6 to 6.5) for the bacterial population pregrown with NaCl than for cells grown without NaCl (pH_i 5 to 5.5) ($P < 0.05$). The protective effect of NaCl was reflected by viable-cell counts at a higher concentration of Incimaxx (0.0031%), where the salt-grown population survived better than the population grown without NaCl ($P < 0.05$). NaCl protected attached cells through drying but not during disinfection. This study indicates that a population of *L. monocytogenes* cells, whether planktonic or attached, is homogenous with respect to sensitivity to an acidic disinfectant studied on the single-cell level. Hence a major subpopulation more tolerant to disinfectants, and hence more persistent, does not appear to be present.**

Listeria monocytogenes is a food-borne, human pathogen that has a remarkable ability to colonize food-processing environments (5, 16, 20, 21, 26, 29). Some *L. monocytogenes* strains can persist for years in food-processing plants (11, 14, 20, 27), and specific molecular subtypes can repeatedly be isolated from the processing environment (29) despite being very infrequent in the outdoor environment (9). This ability to persist has, hitherto, not been linked to any specific genetic or phenotypic trait.

It has been suggested that persistent *L. monocytogenes* strains may be more tolerant or resistant to cleaning and especially disinfectants used in the food industry. Aase et al. (1) found increased tolerance to both benzalkonium chloride and ethidium bromide in *L. monocytogenes* isolates that had persisted for more than 4 years; however, other studies have not been able to link persistence and tolerance to disinfectants (6, 10, 11, 13). We recently compared disinfection sensitivities of persistent and presumed nonpersistent *L. monocytogenes* strains using viable-cell counts and did not find the latter group more sensitive to the two disinfectants Triquart SUPER and Incimaxx DES than persistent strains (13). However, we found that for all subtypes of *L. monocytogenes*, growth with NaCl increased the tolerance of planktonic *L. monocytogenes* cells to Incimaxx DES, whereas spot-inoculated, dried *L. monocytogenes* cells were not protected by NaCl against disinfection.

There is no doubt that *L. monocytogenes* will be completely inactivated at the disinfectant concentrations recommended for use in the food industry; however, the efficiency of the disinfectant is very much influenced by the presence of organic material being inactivated by the presence of food debris. Hence, it is likely that the bacterial cell in a food production environment may be exposed to concentrations at a sublethal level. It is currently not known if treatment with a sublethal concentration of disinfectant affects the entire bacterial population or only attacks a fraction of the cell population, leaving another fraction of cells unaffected. In case of the latter, some bacterial cells may be able to survive the disinfection treatment. The potential presence of such tolerant subpopulations could, ultimately, ensure that the genome is propagated, leading to persistence.

The presence of a more tolerant subpopulation can be determined on the single-cell level. Flow cytometry is a rapid method useable for measurements at the single-cell resolution (22); however, it cannot monitor the same single cells over time. Optical microscopy combined with microfluidic devices that allow measurement of growth of single cells is a useful technique (2), and in situ analyses of the physiological condition of single cells by the fluorescence ratio imaging microscopy (FRIM) technique represents another elegant approach (25). FRIM enables studies of dynamic changes with high

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sensitivity and on the single-cell level in important physiological parameters: e.g., intracellular pH (pH_i). *Listeria* maintains its pH_i within a narrow range of 7.6 to 8 at extracellular pH (pH_{ex}) values of 5.0 to 8.0 (4, 25) and at pH_{ex} 4.0 with the presence of glucose (23). It is believed that viable cells need to maintain a transmembrane pH gradient with their pH_i above the pH_{ex}, and failure to maintain pH_i homeostasis indicates that the bacterial cell is severely stressed and ultimately leads to loss of cell viability. FRIM has been used to determine the pH_i of *L. monocytogenes* after exposure to osmotic and acid stress (7, 23). Also, the dissipation of the pH gradient in *L. monocytogenes* after exposure to different bacteriocins has been determined with FRIM (4, 12). Hornbæk et al. (12) found that treatment with subinhibitory concentrations of leucocin and nisin gave rise to two subpopulations: one consisting of cells with a dissipated pH gradient (ΔpH) and the other consisting of cells that maintained ΔpH, which could indicate phenotypic heterogeneity.

The aim of the present study was to investigate the physiological effects of the disinfectant Incimaxx DES at sublethal and lethal concentrations on single cells and the population level of a persistent *L. monocytogenes* strain to study a possible subdivision of sensitivity in the population. We also addressed the potential protective effect of NaCl against disinfection and compared sensitivities in a population of planktonic and attached bacteria. We applied the in situ technique FRIM and compared the pH_i measurements with the traditional viable-cell-count method.

(Part of the results have been presented at a poster session at the 95th International Association for Food Protection annual meeting in Columbus, OH, 3 to 6 August 2008.)

MATERIALS AND METHODS

Bacterial strains and media. *Listeria monocytogenes* strain N53-1 was isolated from equipment in a fish smokehouse (29). It belongs to the randomly amplified polymorphic DNA type 9 cluster, a molecular subtype that has been persistent for many years in several fish-processing industries (28). Stock cultures were stored at −80°C in 4% (wt/vol) glycerol, 3% (wt/vol) tryptone soya broth (TSB) (CM129; Oxoid, Basingstoke, United Kingdom), 2% (wt/vol) skim milk powder, and 0.5% (wt/vol) glucose. The bacteria were cultivated on brain heart infusion (BHI) agar (CM0225 supplemented with 1.5% agar; Oxoid, Basingstoke, United Kingdom) at 30°C and kept at 4°C for maximum of a month. Subsequent cultures were prepared in TSB supplemented with glucose to a final concentration of 1% (wt/vol), plus in some trials, NaCl was added to a final concentration of 5% (wt/wt). The bacteria were grown overnight at 30°C, diluted 1,000 times, and grown at 30°C for 22 h.

Fluorescence labeling of bacterial cells. Fluorescent labeling of *L. monocytogenes* N53-1 with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Inc., Eugene, OR) was carried out as follows. Bacterial cells were harvested by centrifugation at 14,000 × *g* for 2 min and resuspended in sterile-filtered (pore size, 0.22 μm; GP Express Membrane Filter; Millipore, Bedford, Mass.) citric acid-phosphate buffer solution (pH 7.0) containing 0.37% (wt/vol) citric acid monohydrate and 2.93% (wt/vol) Na_2HPO_4 (19). Glucose and the fluorophor CFDA-SE were added to obtain final concentrations of 10 mM and 10 μM, respectively, and the cells were incubated at 30°C for 30 min. Cells were harvested by centrifugation at 14,000 × *g* for 2 min. For studies of planktonic bacteria, the pellet was resuspended and adjusted in 0.9% NaCl (wt/vol) with 10 mM glucose to an optical density at 600 nm of 0.4 to standardize the biomass. For studies of spot-inoculated and dried *L. monocytogenes* cells, the cells were resuspended and adjusted in sterile growth media to an optical density at 600 nm of 1.0. Bacteria were kept under these conditions for no longer than 2 h.

Immobilization chambers for planktonic bacteria. Planktonic *L. monocytogenes* cells were immobilized on a glass surface essentially as described by Shabala et al. (24). Briefly, a glass coverslip was cleaned with a 70% ethanol–1% HCl mixture, rinsed with sterile Milli-Q water, and dried. Poly-L-lysine solution (0.1% [wt/vol] aqueous solution, P 8920; Sigma Diagnostics, St. Louis, MO) was used to attach cells to the glass surface. One drop (~30 μl) of the poly-L-lysine solution was applied to the coverslip and left for about 5 min to dry. Subsequently, a CoverWell perfusion chamber gasket (C18128; Molecular Probes, Inc., Eugene, OR) was attached and the coverslip and chamber were mounted on a platform designed for the purpose.

Measurement of pH_i of individual bacterial cells. The pH_i of individual *L. monocytogenes* N53-1 bacterial cells was measured by FRIM as described by Guldfeldt and Arneborg (8). 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). Bacterial cells were excited at 488 and 435 nm with an exposure time of 1,000 ms by a Monochromator equipped with a 75-W xenon lamp (Monochromator B; TILL Photonics GmbH, Planegg, Germany). To minimize photobleaching of the stained cells, a 10% neutral-density filter was inserted between the optical fiber and the microscope for the experiments done with the planktonic bacteria, whereas a 2.5% neutral-density filter was used for the experiments with spot-inoculated and dried bacteria. Fluorescence emission was collected with a cool charge-coupled device camera (Coolsnap FX; Photometrics, Roper Scientific), and images were analyzed by using Metavue 6.1 software (Molecular Devices, Dowington, PA). Regions were defined around approximately 40 to 50 individual cells for each experiment by using Metavue. A region near but without the cell was subtracted for each cell as background. The ratio value ($R_{488/435}$) for each cell examined was obtained by dividing the fluorescence intensity at 488 nm (pH-sensitive wavelength) by the fluorescence intensity at 435 nm (pH-insensitive wavelength). The ratio values were transformed to pH_i values by using a calibration curve. To construct this, ethanol (70% [vol/vol]) was added to CFDA-SE-stained *L. monocytogenes* N53-1 cells for 5 min to permeabilize the membrane irreversibly. Subsequently, the bacterial cells were harvested by centrifugation at 14,000 × *g* for 2 min and resuspended in citric acid-phosphate buffers having pH values ranking from 5.0 to 7.7 (19). The calibration curve used for spot-inoculated, dried *L. monocytogenes* N53-1 cells was constructed for bacterium permeabilized and resuspended in TSB with pH values ranging from 5.0 to 9.5.

Preparation of disinfectant solutions. The disinfectant Incimaxx DES (Ecolab Denmark ApS, Valby, Denmark), commonly used in the food industry as a disinfectant and for decalcification, contains a mixture of peroxy acids and hydrogen peroxide as active ingredients. Disinfectant solutions were prepared by dilution in sterilized, demineralized water to obtain concentrations where differences in pH_i were seen. Disinfectant solutions were prepared at 10 times the strength of desired treatment right before use for testing planktonic bacteria and at the desired concentrations for disinfection of spot-inoculated bacteria.

Exposure of planktonic *L. monocytogenes* N53-1 cells to Incimaxx DES. At time zero, 60 μl of a disinfection solution was mixed thoroughly with 540 μl of bacterial cell suspension. Seventy microliters was transferred to the CoverWell perfusion chamber gasket, and microscopic images were acquired every 2.5 min for 20 min in at least three defined positions. Sterile demineralized water was used as the control. Also, to determine the number of viable *L. monocytogenes* cells during disinfection, 40 μl of cell suspension was transferred to 160 μl Dey-Engly (DE) neutralizing broth (281910; Difco, BD, Sparks, France) every 5 min for 20 min. This was done in duplicate. Preliminary experiments showed that DE broth neutralized disinfectants also in higher concentrations of disinfectants than used and that viability of *L. monocytogenes* was unaffected when cells were suspended in DE broth (13). Bacterial numbers were determined by surface plating on BHI agar plates that were incubated at 37°C for 2 days. The complete experiment was repeated twice. Based on a set of preliminary experiments of disinfection sensitivity, *L. monocytogenes* cells were exposed to Incimaxx DES concentrations of 0.0062%, 0.0031%, and 0.0015% (vol/vol). To monitor the pH_{ex} in the bacterial disinfection mixture, pHs were determined every fifth minute for 20 min. Before each experiment, microscopic images of the bacterial cell suspension of *L. monocytogenes* N53-1 were acquired to obtain an initial (t = 0) pH_i value.

Exposure of spot-inoculated and dried *L. monocytogenes* N53-1 cells to Incimaxx DES. Sterile coverslips were placed on a wire screen in a laminar-flow biosafety cabinet. Twenty microliters of CFDA-stained *L. monocytogenes* N53-1 cells suspended in sterile growth media was deposited on each coupon and dried for 20 h in the laminar-flow biosafety cabinet. Spot-inoculated slips were covered with a CoverWell perfusion chamber gasket (C18120; Molecular Probes, Inc., Eugene, OR), and 320 μl Incimaxx DES was added. Sterilized demineralized water was used as the control. To monitor pH_i during disinfection, microscopic images were acquired every 2.5 min for 20 min in three defined positions. To monitor the viability of spot-inoculated, dried bacteria, coverslips were disin-

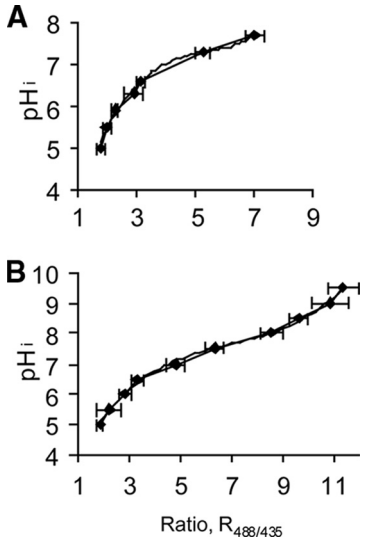

FIG. 1. Relationship between $R_{488/435}$ of the individual cells of *Listeria monocytogenes* N53-1 and pH_i. The pH_i was equilibrated to pH_{ex} by incubating preparations with 70% ethanol and dissolving them at different pH values in buffer (A) or TSB medium (B). The ratio values are averages based on at least 40 single cells. The error bars indicate standard deviations.

fected in parallel in triple determinations for 20 min. After 20 min of incubation, the coupons were transferred with the disinfectant to 10 ml DE neutralizing broth, and bacteria were detached by sonication for 4 min (28-kHz, 2× 150-W sonication bath in a Delta 220; Deltasonic, Meaux, France) (15), vortexed at maximum speed for 15 s and diluted 10-fold serially. Also, three untreated coupons were transferred directly to DE neutralizer broth and sonicated. Cell numbers were determined by surface plating on BHI agar plates that were incubated at 37°C for 2 days. The experiment was repeated at least twice. *L. monocytogenes* was exposed to Incimaxx DES concentrations of 0.062%, 0.031%, and 0.015% (vol/vol). Before each experiment, microscopic images were acquired of a chamber with spot-inoculated and dried *L. monocytogenes* N53-1 cells with 0.9% NaCl added to obtain an initial pH_i value. The pH values of the disinfectants were determined. This equals the pH_{ex} in the bacterial disinfection mixture, as the disinfectant volume used is 16 times the bacterial volume.

Statistical analysis. Statistical comparisons were made between means of pH_i or $\log_{10}$-transformed bacterial counts using Student's t test with a significance level of $P < 0.05$.

RESULTS

Construction of calibration curves. Calibration curves ($R_{488/435}$ versus pH_i) were prepared using ethanol-treated cells of *L. monocytogenes* N53-1 suspended at different pH values in citric acid-phosphate buffer (planktonic bacteria) or TSB medium (attached bacteria) (Fig. 1). The two calibration curves differed at the low-ratio values and were more uniform at the high-ratio values. The probe is very pH sensitive at pH 6.0 to 9.0, while values below pH 6.0 can be difficult to distinguish (Fig. 1). Hence, ratio values less than 1.78 or 1.88 were recorded as pH 5.0 for planktonic and attached bacteria, respectively.

pH_i of planktonic *L. monocytogenes* N53-1 exposed to Incimaxx DES. The viable-cell count and pH_i of planktonic *L. monocytogenes* N53-1 cells grown in TSB with 1% glucose were determined following 20 min of exposure to three different concentrations of Incimaxx DES or demineralized water as control (Fig. 2 and 3). pH_i decreased during the 20-min treatment to pH 5 for all three concentrations of Incimaxx DES,

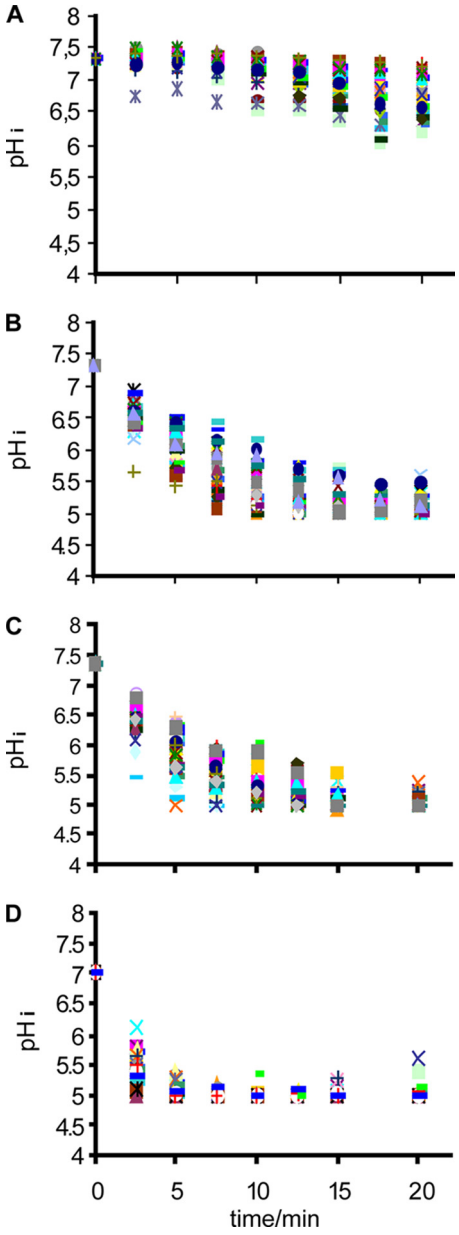

FIG. 2. Change in the pH_i of *Listeria monocytogenes* N53-1 grown in TSB with 1% glucose during disinfection with (A) water (control) or (B) 0.0015%, (C) 0.0031%, or (D) 0.0062% Incimaxx DES. Each point shows the pH_i of a single cell.

indicating that the bacterial cells were stressed (Fig. 2). The rapidity in change in pH_i differed between the three Incimaxx DES concentrations. pH_i decreased to less than 5.5 in 5 min of treatment with a 0.0062% concentration. The change was slower but similar for concentrations of 0.0031% and 0.0015%, where pH_i decreased to less than 5.5 in 10 min. Demineralized water caused only a minor decrease from pH_i 7.33 ± 0.06 to pH_i 6.75 ± 0.28 during the 20-min treatment. The extracellular pH in the bacterial disinfection solutions was 4.0 to 4.1 during 20 min with all three concentrations of Incimaxx DES, including water (data not shown). This indicates that the decrease in pH_i during disinfection is due to Incimaxx DES and not just an

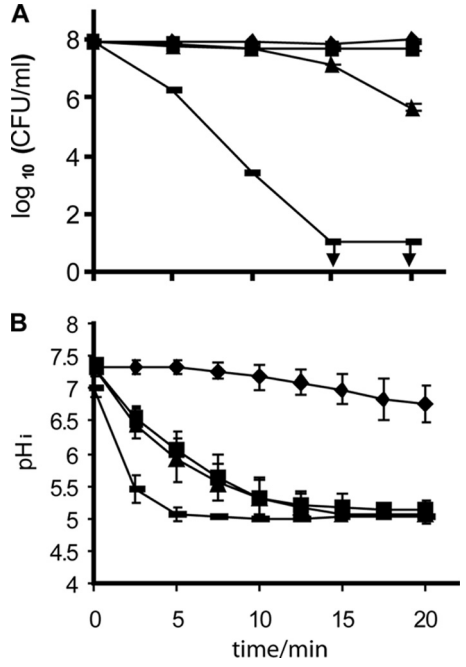

FIG. 3. Viable-cell counts (A) and pHi (B) of *Listeria monocytogenes* N53-1 grown in TSB with 1% glucose and resuspended in 0.9% NaCl with 10 mM glucose during treatment with water (◆) or 0.0015% (■), 0.0031% (▲), or 0.0062% (▬) Incimaxx DES. The viable-cell counts are averages of duplicate determinations. Error bars are based on standard deviations from the duplicate determinations. pH_is are averages based on at least 40 single cells from Fig. 2, and error bars indicate population variation. Arrows indicate that CFU were under the detection limit in the plating assay (10 CFU/ml). The results are representative of two independent experiments.

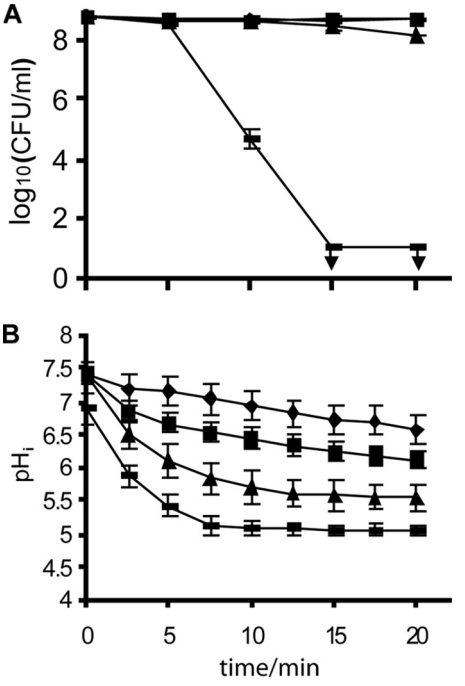

FIG. 4. Viable-cell counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown in TSB with 1% glucose and 5% NaCl and resuspended in 0.9% NaCl with 10 mM glucose during treatment with water (◆) or 0.0015% (■), 0.0031% (▲), or 0.0062% (▬) Incimaxx DES. The viable-cell counts are averages of duplicate determinations. Error bars are based on standard deviations from the duplicate determinations. pH_is are averages based on at least 40 single cells, and error bars indicate population variation. Arrows indicate that CFU were under the detection limit in the plating assay (10 CFU/ml). The results are representative of two independent experiments.

effect of low external pH. The changes in pH_i were similar in all cells measured, indicating that the response of the bacterial population was homogenous (Fig. 2). During the early treatment with Incimaxx DES, the pH_i of the bacterial cells was distributed over 1 to 1.5 pH units; however, the pH_i of the bacterial cells decreased to the same $pH_i \pm 0.5$ and did not split into subpopulations.

The viable-cell counts remained constant over time when *L. monocytogenes* N53-1 was treated with water, indicating that the minor decrease from pH_i 7.33 ± 0.06 to pH_i 6.75 ± 0.28 did not affect viability (Fig. 3). Disinfection with 0.0015% Incimaxx DES caused only a minor decrease in CFU/ml after 20 min, and this decrease was not significantly different ($P > 0.05$) from the counts of bacteria treated with water. Hence, despite the low pH_i of 5 to 5.5 caused by 0.0015% Incimaxx DES, the stressed bacterial cells were able to recover and grow on agar. A more pronounced reduction in viable cells of *L. monocytogenes* N53-1 was seen during treatment with 0.0031% and especially 0.0062% Incimaxx DES. The counts of bacteria treated with 0.0062% were already after 5 min significantly different ($P < 0.05$) from the counts of bacteria treated with water or 0.0015% Incimaxx DES.

The counts of *L. monocytogenes* N53-1 cells pregrown with 5% NaCl did not decrease (CFU/ml) when the bacteria were exposed to 0.0015% Incimaxx DES as compared to water. A slight reduction in viable-cell counts was seen for bacteria exposed for 20 min to 0.0031% Incimaxx (Fig. 3A and Fig. 4A). This protective effect by NaCl was reflected in the pH_i of the bacterial cells, and the pH_i of *L. monocytogenes* N53-1 remained at 6 to 6.5 when treated with Incimaxx DES at 0.0015%. In comparison, the pH_i of 5.5 for bacteria grown without NaCl was significantly lower ($P < 0.05$). The responses of the bacterial population when grown with 5% NaCl were homogenous, and no subpopulations were detected (data not shown).

pH_i of spot-inoculated and dried *L. monocytogenes* N53-1 cells exposed to Incimaxx DES. The response of a bacterial population of *L. monocytogenes* N53-1 cells grown in TSB with 1% glucose and spot inoculated was homogenous during treatment with Incimaxx DES (Fig. 5). However, the pH_i of the population was distributed over a broader range of pH values over time compared to the planktonic population (Fig. 2). There was no indication of formation of subpopulations of cells with increased tolerance. pH_i decreased rapidly to less than 5.5 after 5 min of treatment with 0.062% Incimaxx DES (Fig. 6B). Exposure to 0.031% and 0.015% Incimaxx DES caused the same decline in pH_i from 7.0 to 5.7 during 20 min of treatment. Only a minor decrease in pH_i from 7.0 to 6.5 was seen over time when cells were treated with demineralized water.

The cell numbers of *L. monocytogenes* N53-1 on glass surfaces were equal after treatment with water or 0.015% or

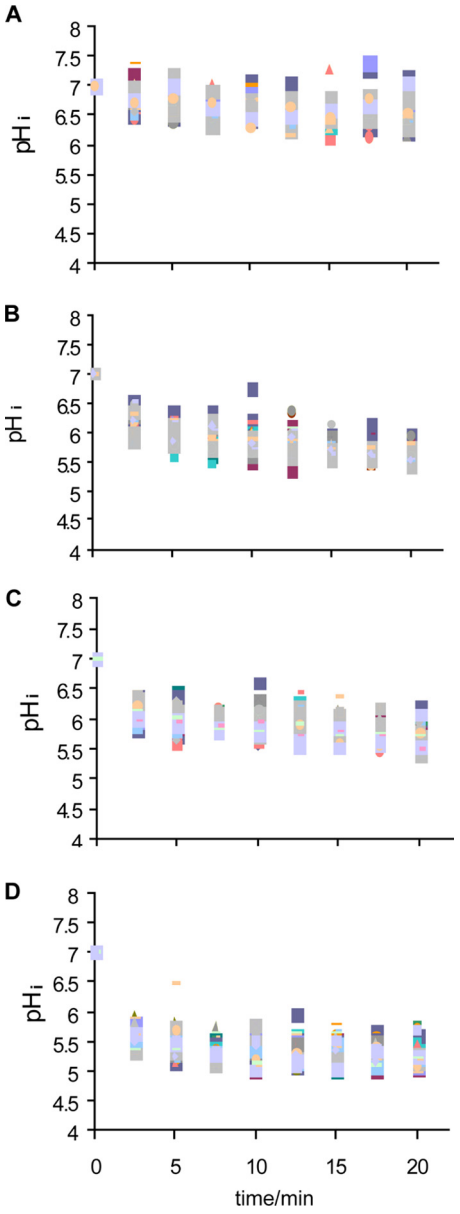

FIG. 5. Change in the pH_i of *Listeria monocytogenes* N53-1 grown, spot inoculated, and dried in TSB with 1% glucose during disinfection with water (control) (A) or 0.015% (B), 0.031% (C), or 0.062% (D) Incimaxx DES. Each point shows the pH_i of a single cell.

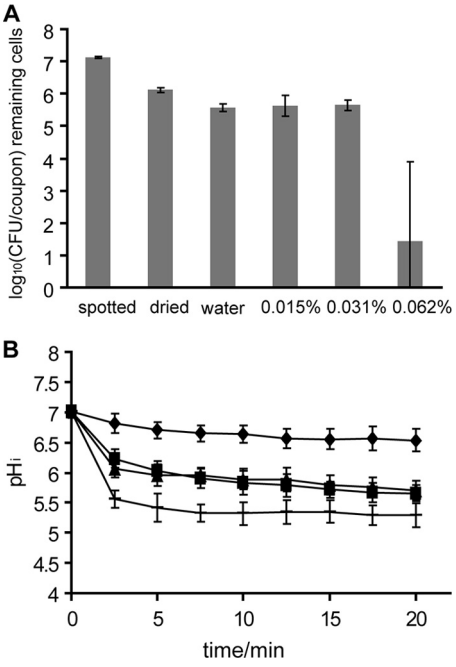

FIG. 6. Viable-cell counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown, spot inoculated, and dried in TSB with 1% glucose during treatment with water (◆) or 0.015% (■), 0.031% (▲), or 0.062% (▬) Incimaxx DES. The viable-cell counts are averages of triplicate determinations. Error bars are based on standard deviations from the triplicate determinations. pH_is are averages based on at least 40 single cells, and error bars indicate population variation. The results are representative of two independent experiments.

0.031% Incimaxx DES. However, 0.062% Incimaxx DES caused a marked decrease in bacterial numbers (Fig. 6A).

Addition of NaCl to *L. monocytogenes* N53-1 caused more cells to survive the drying process, since the numbers of CFU/coupon were significantly higher ($P < 0.05$) than those of *L. monocytogenes* N53-1 grown, spot-inoculated, and dried in TSB with 1% glucose (Fig. 6A and Fig. 7A). This indicates that NaCl protects the bacterial cells during drying. Also, this protective effect could be seen from the pH_i measurement during treatment with water, since the pH_i of *L. monocytogenes* N53-1 grown and spotted with 5% NaCl was 7.1 after 20 min, while it was 6.5 for *L. monocytogenes* N53-1 grown and spotted without NaCl. This indicates that preculture with NaCl enable the bacterial cells to keep the pH_i more constant in water. However, when disinfecting *L. monocytogenes* N53-1 cells dried with 5% NaCl, the number of remaining bacterial cells decreased as rapidly as the non-NaCl-grown cells with increasing concentrations of Incimaxx DES, indicating that preculture and spotting with NaCl render the attached, dried bacterial cells more sensitive than or as sensitive to Incimaxx DES as N53-1 cells grown and spotted without NaCl.

DISCUSSION

Some subtypes of *L. monocytogenes* are able to persist in food-processing plants for many years (11, 14, 20, 27, 29), and this is a major food safety issue as they constitute a constant reservoir of food product contamination. The seriousness of this is highlighted by a recent (summer 2008) outbreak of listeriosis in Canada that has been caused by meat products contaminated with *L. monocytogenes* on two processing lines in a meat factory. The ability to persist has, hitherto, not been linked to any specific genetic or phenotypic trait. It has been hypothesized that persistent *L. monocytogenes* strains were less susceptible to disinfection than nonpersistent strains; however, most studies have not been able to confirm this hypothesis (6, 10, 11, 13). However, the persistent strains may reside in the processing environment due to the constant maintained tolerant subpopulation, and such heterogeneity at the single-cell level would be masked in a study of a sensitivity and killing kinetics in the complete bacterial population. In the present

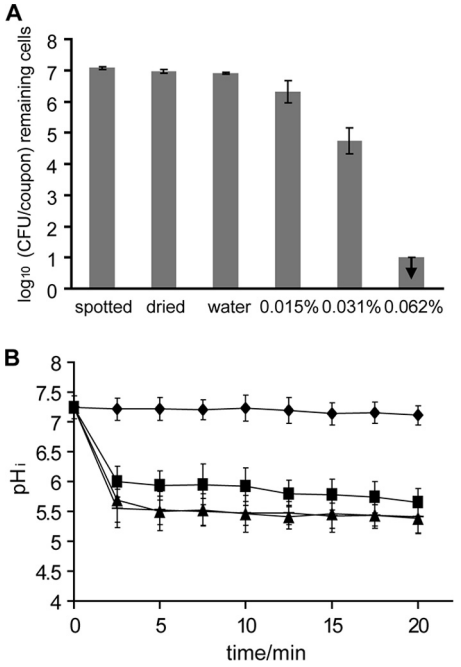

FIG. 7. Viable-cell counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown, spot inoculated, and dried in TSB with 1% glucose and 5% NaCl during treatment with water (◆) or 0.015% (■), 0.031% (▲), or 0.062% (▬) Incimaxx DES. The viable-cell counts are averages of triplicate determinations. Error bars are based on standard deviations from the triplicate determinations. pH_is are averages based on at least 40 single cells, and error bars indicate population variation. Arrows indicate that CFU were under the detection limit in the plating assay (100 CFU/coupon). The results are representative of two independent experiments.

study, we have for the first time used FRIM to study the physiological effect of disinfectants at the single-cell level by measuring pH_i. We did not, however, find an indication that sublethal concentrations of the disinfectant studied caused division of the population in subpopulations.

As mentioned, the present study is to our knowledge the first study using measurement of pH_i to detect the appearance of subpopulations during disinfection. The technique detected the single-cell physiological response to the stress factor at concentrations where the determination of bacterial density (CFU/ml or CFU/coupon) did not reveal any effects. Other studies have monitored pH_i over time for exponential- and stationary-phase cells of *L. monocytogenes* during exposure to benzalkonium chloride (17). However, in these studies, the pH_i was measured on a population basis using spectrophotometry and single cells could not be monitored. In the present study, we did not detect any more tolerant subpopulation, neither in the planktonic nor in the attached population, since the decrease in pH_i was very homogenous for all cells over time. Earlier, the FRIM method had successfully been used to detect subpopulations in *L. monocytogenes* cultures exposed to bacteriocins (12). Similarly, *L. monocytogenes* cells previously grown on agar plates were heterogeneous with respect to sensitivity to nisin when measuring pH_i, and some of the cells appeared to be more tolerant than others (4).

A pH_i value of 5.0 to 5.5 is the lower limit of the sensitivity for CFDA (3), which was also seen from the calibration curves. As the external pH is around 4, the pH_i measurement can be used for information on stress conditions of the cells but not on viability. Another dye with higher sensitivity at lower pH could have been used instead. However, the low-pH-sensitive dye would not cover the neutral-pH range, leading to loss of information. Instead we combined the pH_i measurement with viability measured as CFU on agar plates.

We found that growth with a typical food component, NaCl, protected the bacteria against drying and disinfection. When using pH_i as a measure of bacterial response, the protective effect was seen at treatments with 0.0015% Incimaxx, whereas it was reflected by viable-cell counts at a higher concentration of Incimaxx (0.0031%), in accordance with earlier results (13). As pH_i decreased very rapidly to below 5.5 during disinfection with 0.0031% Incimaxx DES, one may speculate if using a probe more sensitive in the lower-pH area could have indicated a protective effect of NaCl. Bacteria that were spot inoculated and dried on surfaces were only protected by NaCl during the drying stage but not when exposed to disinfectants. This is in agreement with our previous data using colony counts to measure the effect of disinfectants (13). A higher concentration of Incimaxx DES was needed to eliminate attached *L. monocytogenes* cells compared to planktonic bacteria cells. However, this is more likely due to the higher biological load introduced by the setup than to increased tolerance of the attached bacteria per se.

In the present study, *L. monocytogenes* survived a pH_i of <5.5 for more than 10 min as planktonic cells during disinfection with 0.0015% Incimaxx DES, and cell viability was not significantly different from the viability in water. Shabala et al. (23) measured a pH_i of ≤ 5 after 2 h for *L. monocytogenes* maintained at pH_{ex} 3.0, and the cells remained viable as the organism recovered immediately and remained constant at pH_i 7.3, when returning to pH_{ex} 6.0. Hence, the ability of this organism to sustain a low pH_i, even though it is critical for many cellular processes, such as DNA transcription, protein synthesis, and enzyme activities, may contribute to the survival of *L. monocytogenes* in acidified environments.

pH_i was a more sensitive measure of adverse effects on *L. monocytogenes* than viability (CFU). Thus, a concentration of Incimaxx DES (0.0015%) that did not significantly affect cell counts had a marked effect on pH_i (Fig. 3). Similarly, Luppens et al. (18) found that the ability to maintain a pH gradient was largely affected by benzalkonium chloride and hydrogen peroxide before a major loss in viability (according to plate counts) was detected. This indicates that antibacterial components may clearly stress bacterial cells even at levels where no effect is seen on viable-cell counts. Measurements of pH_i allow an online indication of the physiological status of bacterial cells and can be used to monitor both individual bacterial cells and a population of bacteria. Due to the high sensitivity of the pH_i measurement, it is useful for determination of subinhibitory concentrations of disinfectants: i.e., concentrations that do stress the bacteria by decreasing pH_i but do not affect viability. This is highly useful for further studies of the impact of subinhibitory stress on *L. monocytogenes* in relation to, for example, gene expression and virulence. Furthermore, the method may be useful for studying subinhibitory concentrations of antibiotics.

In conclusion, we have shown that the response of a persistent strain of *L. monocytogenes*, whether planktonic or attached, is homogenous on the single-cell level to an acidic disinfectant; hence, subpopulations do not appear. It is not likely that the persistent strain of *L. monocytogenes* survives in the production environment due to presence of a more tolerant subpopulation. The pH_i measurement is useful for determination of subinhibitory concentrations of disinfectants and is relevant for further studies of the impact of subinhibitory stress on *L. monocytogenes.*

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