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Influence of Sublethal Concentrations of Common Disinfectants on Expression of Virulence Genes in *Listeria monocytogenes*▽

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***Listeria monocytogenes* is a food-borne human pathogen that causes listeriosis, a relatively rare infection with a high fatality rate. The regulation of virulence gene expression is influenced by several environmental factors, and the aim of the present study was to determine how disinfectants used routinely in the food industry affect the expression of different virulence genes in *L. monocytogenes* when added at sublethal concentrations. An agar-based assay was developed to screen the effect of disinfectants on virulence gene promoter expression and was validated at the transcriptional level by Northern blot analysis. Eleven disinfectants representing four different groups of active components were evaluated in this study. Disinfectants with the same active ingredients had a similar effect on gene expression. Peroxy and chlorine compounds reduced the expression of the virulence genes, and quaternary ammonium compounds (QAC) induced the expression of the virulence genes. In general, a disinfectant had similar effects on the expression of all four virulence genes examined. Northern blot analyses confirmed the downregulation of *prfA* and *inlA* expression by Incimaxx DES (a peroxy compound) and their upregulation by Triquart Super (a QAC) in *L. monocytogenes* EGD. Hence, sublethal concentrations of disinfectants routinely used in the food industry affect virulence gene expression in the human pathogen *L. monocytogenes*, and the effect depends on the active components of the disinfectant. From a practical perspective, the study underlines that disinfectants should be used at the lethal concentrations recommended by the manufacturers. Further studies are needed to elucidate whether the changes in virulence gene expression induced by the disinfectants have impact on virulence or other biological properties, such as antibiotic resistance.**

Listeria monocytogenes is a food-borne, facultative intracellular pathogen that can cause invasive listeriosis in immunocompromised individuals, pregnant women, infants, and the elderly. The disease is relatively rare (0.3 cases per year per 100,000 inhabitants in the European Union), but it is associated with a high fatality rate (25 to 30%) (11).

L. monocytogenes can cross the intestinal barrier, the blood-brain barrier, and the placental barrier, and it invades and replicates in both phagocytic and nonphagocytic cells (47). The ability to invade and to survive and multiply within eukaryotic cells is determined by a number of chromosomal genes, most of which are found on the 9-kb pathogenic island known as *Listeria* pathogenicity island 1 (LIPI-1) but also on other places on the chromosome. These virulence genes encode products involved in adherence to and internalization by the host cell (*inlA*, *inlB*, and *inlC*), escape from the vacuoles (*hly*, *plcA*, and *plcB*), intracellular replication (*htp*), and cellular movement (*actA*). The major transcriptional factor which regulates the expression of these virulence genes, including its own transcription, is the 27-kDa polypeptide PrfA (5, 8, 12, 18, 27, 32).

The regulation of virulence gene expression by the PrfA protein is dependent on the concentration and activity of the protein but also on the configuration of the virulence gene promoters. The PrfA protein facilitates specific binding to its target site, the so-called PrfA box, which partially overlaps the promoter region, and affinity is weakened when this target sequence diverges from the perfect palindromic sequence (17, 44, 49).

The regulation of PrfA and virulence gene expression is influenced by several environmental factors. One example is the temperature-dependent control of translation of the *prfA* messenger, which is processed only at 37°C and not at 30°C (22, 26). Another example is the repression of virulence gene expression in response to high concentrations of fermentable carbohydrates (35), and a third example is the induction of virulence gene expression observed when *L. monocytogenes* is grown with activated charcoal, due to the absorption of an autorepressor (13, 40). Less-well-described physicochemical parameters also affect virulence gene expression. Nutrient stress conditions limiting growth induced gene expression of *prfA*, *plcA*, *hly*, and *actA* (3, 45), and high osmolarity induced expression of *hly* (37). On the other hand, growth at pH 6 to 6.8 repressed *hly* expression (2).

L. monocytogenes is frequently detected in the food processing environment, where it has a remarkable ability to persist (7, 29, 34, 38, 50). An efficient cleaning and disinfection process is essential in preventing contamination of food products with *L. monocytogenes* during processing. However, the disinfection process is not always adequately performed, or organic debris may inactivate the disinfectant; hence, the bacteria may be exposed to only sublethal concentrations and survive.

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TABLE 1. Effect of different disinfectants on expression of different virulence genes of *Listeria monocytogenes*

Type of disinfectant	Designation (pH)	Company	Concn (%)	Regulation of gene fusion[a]				
				prfA-lacZ	*plcA-lacZ*	*inlA-lacZ*	Δ*prfA inlA-lacZ*	*hly-lacZ*
Peroxy compounds	Incimaxx DES (acid)	Ecolab, Valby, Denmark	1.25–40	−	−	−	−	−
	DES foam PAA (acid)	ITW Novadan APS, Kolding, Denmark	0.31–10	−/+	−/+	−/+	−/+	−/+
Chlorine	P3-hypochloran (alkaline)	Ecolab	3.13–100	−	−	−	−	−
	Desinfect CL (alkaline)	ITW Novadan APS	3.13–100	−	−	−/+	−/+	−
	Chlorrengøring (alkaline)	Samson Enviro Industries, Kvistgård, Denmark	3.13–100	−	−	−	−	−
Triclosan, ethanol	Overfladedesinfektion (neutral)	Pro-ren A/S, Holbaek, Denmark	0.19–6	+	None	+	+	+
Quaternary ammonium compounds	Triquart Super (alkaline)	Ecolab	0.0063–0.2	+	+	+	+	+
	Multidesinfektion (neutral)	Samson Enviro Industries	0.0063–0.2	+	+	+	+	+
	Desinfect Maxi (neutral)	ITW Novadan APS	0.0063–0.2	+	+	+	+	+
	Desinfect Ultra (acid)	ITW Novadan APS	0.0063–0.2	+	+	+	+	+
	Desinfect Alka (alkaline)	ITW Novadan APS	0.0063–0.2	+	+	+	+	+

[a] +, induction of expression of the virulence gene; −, reduction of expression of the virulence gene; −/+, reduction followed by induction of expression of the virulence gene; none, neither induction nor reduction of expression of the virulence gene.

L. monocytogenes that resides in food processing environments may adapt to disinfectants after repeated exposure (1, 46); however, few studies have investigated how low concentrations of disinfectants affect the physiology of *L. monocytogenes* at the gene expression level. Recently, we found that exposure of *L. monocytogenes* to sublethal concentrations of a disinfectant stressed the cell, measured as a decrease in intracellular pH (24), and Ryan et al. (42) found that the *sigB* gene was upregulated in the presence of quaternary ammonium compounds (QAC) and sodium dodecyl sulfate (SDS), which are common components of industrial cleaning agents. Hence, an element in assessing the risk of *L. monocytogenes* contaminating a food product is to determine if food environment stress factors such as sublethal concentrations of disinfectant affect expression of virulence genes in *L. monocytogenes*.

The aim of the present study was to determine how disinfectants used routinely in the food industry affect the expression of different virulence genes in *L. monocytogenes* when added at sublethal concentrations. An agar-based assay was developed and applied for screening the effect of disinfectants on virulence gene promoter expression and was validated at the transcriptional level by Northern blot analysis.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *Listeria monocytogenes* EGD and the Δ*prfA* mutant were obtained from Werner Goebel (Biozentrum, University of Würzburg). Strains of *L. monocytogenes* EGD with *lacZ* fusions to the *hly*, *plcA*, *prfA*, and *inlA* promoters in the vector pTCV-lac were from the laboratory collection at the Department of Veterinary Disease Biology, Faculty of Life Science, University of Copenhagen (25). Stock cultures were stored at −80°C in 15% (wt/vol) glycerol. The bacteria were cultivated on brain heart infusion (BHI) agar (CM0225 [Oxoid, Basingstoke, United Kingdom] supplemented with 1.5% agar) at 37°C for 24 to 48 h. One colony was cultured in BHI broth and incubated with shaking (180 rpm) at 37°C overnight, diluted 1,000-fold, and grown at 37°C and 180 rpm for 18 h. Kanamycin was added to growth media for the strains of *L. monocytogenes* EGD with *lacZ* fusions at a final concentration of 50 μg/ml.

Preparation of disinfectant solutions. Eleven disinfectants commonly used in the food industry were tested in this study (Table 1). The disinfectants represent four different groups of active compounds. Disinfectants were prepared in 2-fold dilutions in sterilized, demineralized water to obtain concentrations at which inhibition zones with appropriate sizes (i.e., zones did not merge) were seen in the agar assay (described below).

Agar-based screening assay. An agar-based assay was developed on the basis of an existing assay for *Staphylococcus aureus* (A. Nielsen, K. F. Nielsen, T. O. Larsen and H. Ingmer, unpublished data). BHI agar and charcoal-supplemented BHI (BHI-AC) (BHI with addition of 0.013% or 0.025% activated charcoal) was melted and tempered to 44°C. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (150 μg/ml) and kanamycin (50 μg/ml) were added. One milliliter of outgrown bacterial culture (i.e., grown overnight, diluted 1,000-fold, and grown for 18 h) with a promoter-*lacZ* fusion, diluted 1,000-fold in 0.9% NaCl, was mixed thoroughly with 25 ml of medium in a petri dish (diameter, 9 cm), and the plates were dried in a laminar air flow (LAF) bench for 45 min. Seven wells (4-mm diameter) were made in each plate with a sterilized drill, and 30 μl of disinfectant at different concentrations was added to each well. Water was used as control. The plates were incubated at 37°C for 48 h. Each disinfectant was tested in at least two independent experiments with all strains containing promoter-*lacZ* fusions. In each experiment, all strains were screened in BHI and BHI-AC. Strains containing plasmids p*prfA-lacZ*, p*hly-lacZ*, and p*inlA-lacZ* were screened in 0.013% BHI-AC, while the strain containing plasmid p*plcA-lacZ* were screened in 0.025% BHI-AC in order to further induce promoter activity (see below).

Construction of promoter-*lacZ* fusions in a Δ*prfA* strain. To study if the responses of the different virulence gene promoters were influenced by the PrfA protein, the *lacZ* fusions were transformed into the Δ*prfA L. monocytogenes* EGD strain. Listerial plasmid DNA was purified from strains containing plasmid p*hly-lacZ*, p*inlA-lacZ*, or p*plcA-lacZ* using the Qiaminiprep kit (Qiagen). The plasmid isolation procedure was modified by incubating the cell suspension in p1 buffer containing lysozyme (9 mg/ml) for 1 h at 37°C. Competent Δ*prfA* cells were prepared as described by Park and Stewart (39) with slight modifications. Briefly, 3 ml of an overnight culture was cultured in 150 ml BHI with 0.5 M sucrose at 37°C with shaking (200 rpm) until an optical density at 600 nm (OD_{600}) of 0.2 was reached. Penicillin G (10 μg/ml) was added and the incubation continued for a further 2 h (100 rpm). Cells were harvested (3000 × *g*, 10 min, 4°C) and washed three times with sucrose electroporation buffer (1 mM HEPES [pH 7.0], 0.5 M sucrose). The final cell pellet was resuspended in 500 μl ice-cold glycerol solution (1 mM HEPES, 0.5 M sucrose, 15% glycerol) and stored at −80°C prior to use. Cells (40 μl) and 1 μl plasmid DNA were mixed, and electroporation was performed (25 μF, 2.5 kV, 200 Ω). After the pulse, 1 ml prewarmed BHI (37°C) was added, mixed with the cells, and transferred to 15 ml BHI. After 3 h of incubation at 30°C with shaking (150 rpm), cells were spread on BHI plates with kanamycin (5 μg/ml).

RNA extraction and Northern hybridization. An outgrown culture (i.e., grown overnight, diluted 1.000-fold, and grown for 18 h) of *L. monocytogenes* EGD was

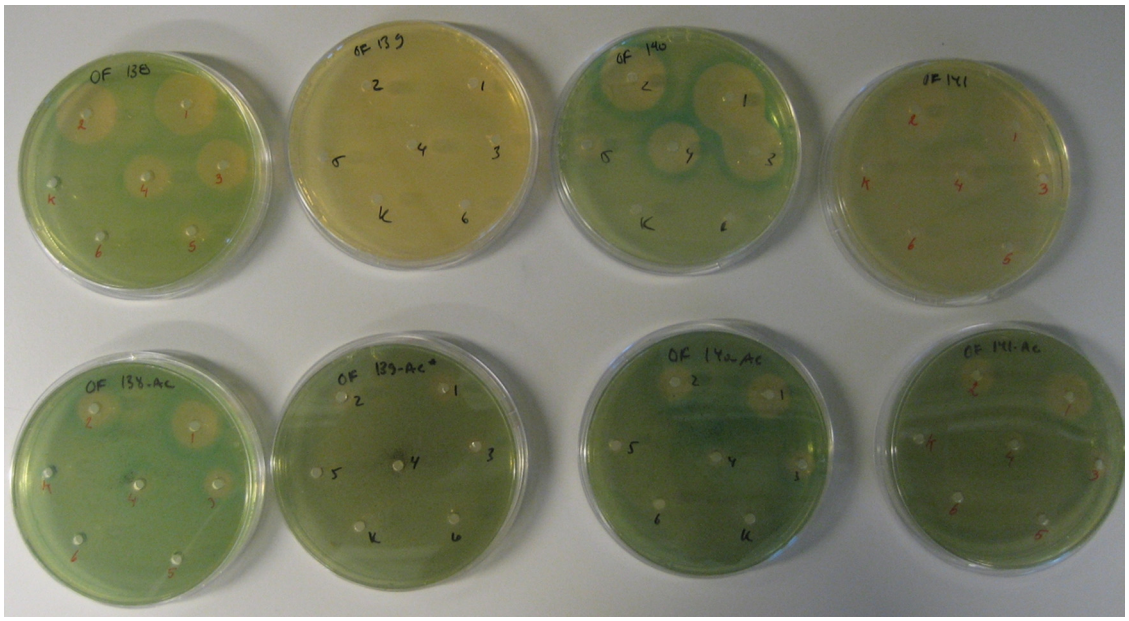

FIG. 1. *Listeria monocytogenes* with *lacZ* fusions with p*prfA*, p*plcA*, p*inlA*, and p*hly* promoters (from left to right, respectively) diluted 1,000-fold and cast in BHI (upper row) and BHI-AC (lower row). Overfladedesinfektion was added at decreasing concentrations in wells 1 to 6 from right to left. Water was added as control (K) and did not alter the color of the agar. The results presented are representative of two independent experiments.

diluted 100-fold in BHI and grown to an OD_{600} of 0.4. The culture was split and centrifuged at 3,000 × *g* for 10 min, and the pellet was resuspended in BHI-AC broth (0.2% charcoal) with 0.25% or 0.125% Incimaxx DES or in BHI broth with 0.0031% or 0.0016% Triquart Super. The bacterial cells were resuspended to an OD_{600} of 0.4, and 60 ml of each culture was transferred to a 300-ml Erlenmeyer flask. Demineralized water was used for the control cultures. Cells were grown at 37°C with shaking (130 rpm). Samples were taken after 15, 30, 60, and 180 min of growth with the disinfectant for RNA extraction. RNA was stabilized by the addition of 2 volumes RNA Protect (Qiagen), and bacterial cells were harvested by centrifugation at 5,000 × *g* for 10 min and stored at −80°C. Bacterial numbers were determined by surface plating on BHI agar plates after 0, 60, and 180 min of growth. BHI agar plates were incubated at 37°C overnight. To extract RNA, the bacterial cells were lysed using the Fast Prep FP120 instrument (BIO101, ThermoSavent) for 45 s at speed 6. The cells were kept on ice for 1 min and then lysed again. The treatment and cooling on ice were repeated three times. Total RNA was extracted from the cells using the RNeasy mini kit (Qiagen) according to the manufacturer's directions. Analysis of transcripts was performed as described by Frees et al. (15) with slight modifications. Briefly, total RNA was quantified with a Nanodrop 2000 (Thermo Fisher Scientific), and 5 μg of RNA of each preparation was loaded onto a 1% agarose gel and separated in 10 mM sodium phosphate buffer. RNA was transferred to a positively charged nylon membrane (Amersham HybondN; GE Health Care) by capillary blotting. Hybridization probes were generated by PCR from chromosomal DNA of *L. monocytogenes* EGD using specific primers for the *prfA* gene (1F, 5′TAA CCA ATG GGA TCC ACA AG-3′; 1R, 5′TGC TAA CAG CTG AGC TAT GTG-3′) and the *inlA* gene (1,5F, 5′ATC GAT GGA GTG GAA TAC TT-3′; 1,5R, 5′ GTG CCT ATA TCT TTT AAC TGG TTA C-3′). Probes were labeled with [^{32}P]dCTP using Ready-to-Go DNA-labeling beads (Amersham Biosciences). RNA from at least two independent experiments was analyzed.

RESULTS

Screening of disinfectants in agar assay. To determine how different disinfectants affect the expression of virulence genes in *L. monocytogenes*, we designed an agar-based screening assay in which virulence gene expression is monitored as β-galactosidase production from *L. monocytogenes* derivatives carrying *lacZ* fused to the promoters of each of the known virulence factor genes *hly*, *plcA*, *prfA*, and *inlA* (25). In order to be able to monitor changes in virulence gene expression, we chose to use a growth medium that supports a low level of virulence gene expression, as is the case with BHI and BHI-AC. When we incorporated the *L. monocytogenes* strains with *lacZ* fusions in the agar plates, a dense growth was observed. The *L. monocytogenes prfA-lacZ* strain turned a strong blue in BHI or BHI-AC agar plates, indicating that the *prfA* gene was expressed during growth in BHI and that charcoal did not visually induce the *prfA* promoter further (Fig. 1, two leftmost plates). The *lacZ* gene of the *inlA*- and *hly-lacZ* fusions was expressed in BHI, but addition of charcoal caused an even more intense blue color, indicating an induction of the promoters by charcoal (Fig. 1, four rightmost plates). In contrast, addition of activated charcoal was necessary to obtain visible *lacZ* expression of the *plcA-lacZ* fusion, and the amount of charcoal needed to obtain a sufficient blue color was 2-fold higher than that for the other strains (Fig. 1, two plates in the second column from the left).

After having confirmed that differences in virulence gene expression could be monitored in agar plates, disinfectants were added to wells formed in the agar, and each of the *lacZ* fusion strains was examined in BHI and BHI-AC agar. Disinfectants were added at inhibitory concentrations, and for all strains the size of the inhibition zones decreased as expected with decreasing concentration of disinfectant (Fig. 2 and data not shown). Also, each well represents a screening of disinfectant concentrations as the disinfectant dilutes outward from each well. For some disinfectants, white colonies were seen outside of the inhibition zones, indicating that those disinfectants at sublethal concentrations reduced the expression of the virulence gene (Fig. 2A). For other disinfectants, colonies out-

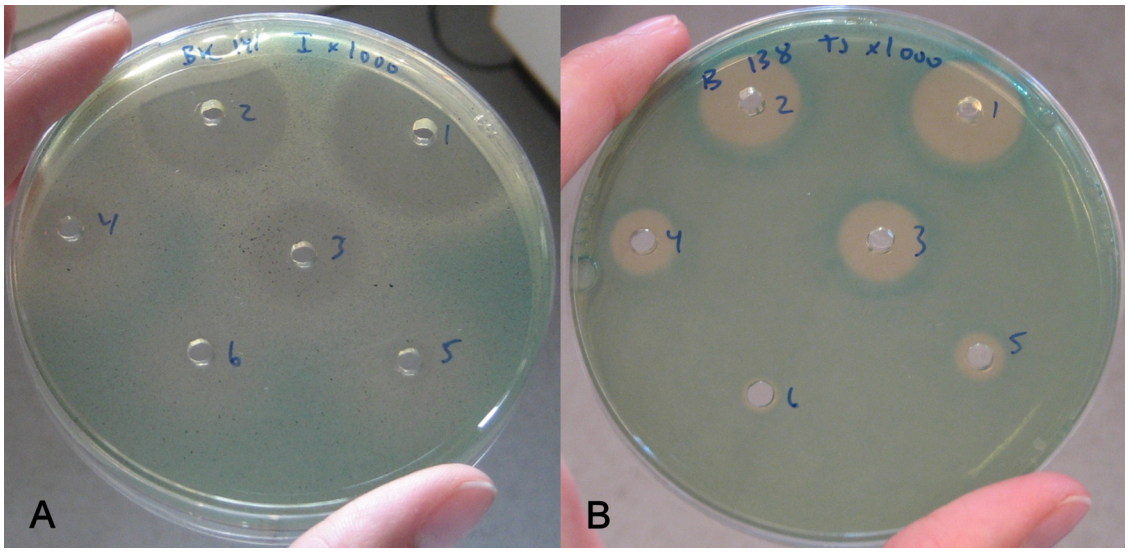

FIG. 2. (A) *Listeria monocytogenes* with a *lacZ* fusion with the p*hly* promoter diluted 1,000-fold and cast in BHI-AC. Incimaxx DES was added at decreasing concentrations in wells 1 to 6. (B) *L. monocytogenes* with a *lacZ* fusion with the p*prfA* promoter diluted 1,000-fold and cast in BHI. Triquart Super was added at decreasing concentrations in well 1 to 6. The results are representative of two independent experiments.

side the inhibition zones were of a more intense blue color than the colonies further away from the inhibition zones, indicating that those disinfectants at sublethal concentrations induced the expression of the virulence gene (Fig. 2B). No inhibition or effect on gene expression was seen when water was added to the well (control) (Fig. 1).

In total, 11 disinfectants representing four groups of active components were evaluated in the present study (Table 1). They are all commonly used in the food industry and are supplied by different manufacturers. Disinfectants with the same active ingredients in general caused similar effects on gene expression. Peroxy and chlorine compounds reduced the expression of the virulence genes, and quaternary ammonium compounds (QAC) induced the expression of the virulence genes. However, for DES foam PAA, white colonies were seen around the inhibition zone and followed by an intense blue zone, which could indicate that the disinfectant at a sublethal concentration caused a reduction in the expression of the virulence gene but at a lower concentration caused induction of the virulence gene expression.

In general, a disinfectant compound had the same effect on the expression of all four virulence gene promoters (Table 1). However, Desinfect CL caused downregulation of three fusions (*prfA*, *plcA*, and *hly*), but DES foam PAA caused a combined down- and upregulation of *inlA*. Similarly, the compound Overfladedesinfektion induced only three promoters (*prfA*, *inlA*, and *hly*) and had no visible effect on the *plcA* promoter.

Screening of Δ*prfA lacZ* fusion strains. Since many of the virulence genes in *L. monocytogenes* are controlled by PrfA, we determined if the effect of the different disinfectants on virulence gene expression was dependent on PrfA. To study this, the plasmids containing the reporter gene fusions (p*plcA-lacZ*, p*inlA-lacZ*, and p*hly-lacZ*) were transformed into the *prfA* deletion mutant.

The three Δ*prfA* promoter-*lacZ* transformants were all screened in the agar assay. Expression of *inlA* was induced in BHI, and addition of charcoal increased also the expression in the absence of PrfA. However, there was no visible expression of *plcA* or *hly* in the *prfA* deletion strains during growth in BHI or BHI-AC, nor could growth of the two strains in diluted BHI (1:2, 1:4, and 1:8) agar (1.5%) or diluted tryptic soy (1:7) agar (1.5%) with or without 0.013% charcoal induce the promoters. Hence, only the Δ*prfA* p*inlA-lacZ* strain could be screened with the disinfectants in our agar assay.

The influence of the 11 disinfectants on gene expression in the Δ*prfA* p*inlA-lacZ* strain was determined. All disinfectants had the same effect on this strain as on the wild-type p*inlA-lacZ* strain, which contains the *prfA* gene (Table 1). When comparing the intensity of the background color as well as the blue induction zones or the white reduction zones, no clear difference was seen between the Δ*prfA* p*inlA-lacZ* strain and the p*inlA-lacZ* strain. This indicates that the up- or downregulation of the *inlA* promoter by disinfectants was not primarily caused by PrfA.

Northern blot analysis of virulence gene expression in the presence of disinfectant. To study whether the effect of disinfectants on the virulence gene promoter was indeed a true gene induction or repression effect, we determined the effect of sublethal concentrations of disinfectants on the transcript levels of the *prfA* and *inlA* genes. Northern blot analyses were performed with the wild-type *L. monocytogenes* EGD and the two disinfectants Incimaxx DES and Triquart Super, which down- and upregulate gene expression in the agar assay, respectively. Both disinfectants were tested at sublethal and noninhibiting concentrations (Fig. 3) to ensure that the growth rates were similar so the total RNA concentrations per bacterial cell were comparable.

A marked downregulation of transcript levels was seen for *prfA* and *inlA* compared to EGD grown with water when *L. monocytogenes* EGD was grown with 0.25% and 0.125% Incimaxx DES (Fig. 4). The *plcA-prfA* transcript of 2.1 kb was downregulated at 15, 30, 60, and 180 min of growth with both

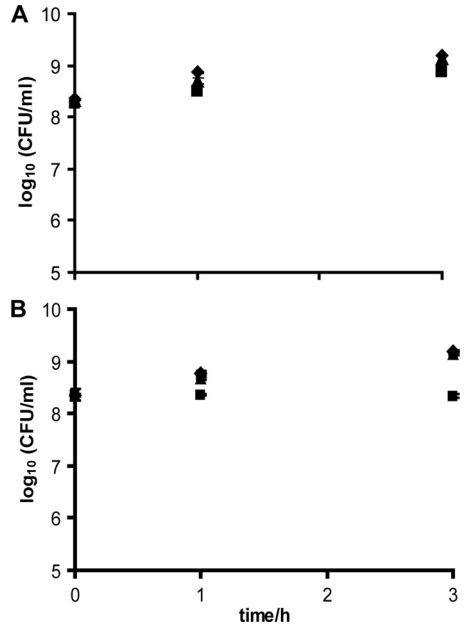

FIG. 3. (A) Growth of *Listeria monocytogenes* EGD in BHI-AC (0.2% charcoal) broth with water (control) (◆) or 0.25% (■) or 0.125% (▲) Incimaxx DES. (B) Growth of *Listeria monocytogenes* EGD in BHI broth with water (control) (◆) or 0.0031% (■) or 0.0016% (▲) Triquart Super. The results are representative of two independent experiments.

0.125% (lanes B, E, H, and K) and 0.250% (lanes C, F, I, and L) Incimaxx DES compared to the control (lanes A, D, G, and J) (Fig. 4A). Similarly, the monocistronic *prfA* transcripts of 0.8 and 0.9 kb were downregulated compared to controls with water. The downregulation was more pronounced in trials with 0.25% Incimaxx DES than in those with 0.125% Incimaxx DES. Both *inlA* (2.9 kb) and *inlAB* (5 kb) transcripts were detected after 15 min of growth in the control, whereas only the *inlA* transcript was detected during further growth (Fig. 4B). However, only very weak transcripts of *inlA* were detected

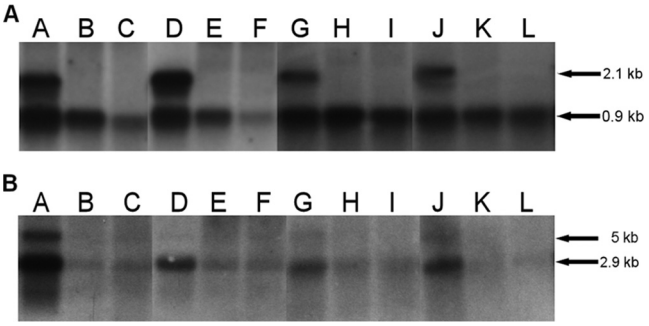

FIG. 4. *Listeria monocytogenes prfA* (A) and *inlA* (B) transcription measured by Northern blotting using RNA isolated from *L. monocytogenes* EGD grown in BHI-AC (0.2% charcoal) for 15 min (lanes A, B, and C), 30 min (lanes D, E, and F), 60 min (lanes G, H, and I), or 180 min (lanes J, K, and L) with water (control) (lanes A, D, G, and J) or 0.125% (lanes B, E, H, and K) or 0.250% (lanes C, F, I, and L) Incimaxx DES. The arrows show the *plcA-prfA* (2.1-kb), *prfA* (0.8- and 0.9-kb), *inlAB* (5-kb), and *inlA* (2.9-kb) transcripts. The results are representative of two independent experiments.

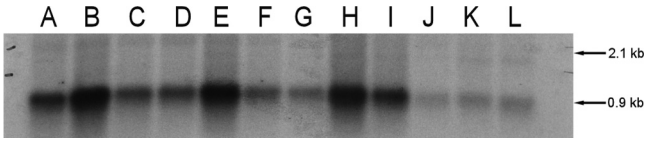

FIG. 5. *prfA* transcription measured by Northern blotting using RNA isolated from *Listeria monocytogenes* EGD grown for 15 min (lanes A, B, and C), 30 min (lanes D, E, and F), 60 min (lanes G, H, and I), or 180 min (lanes J, K, and L) with water (control) (lanes A, D, G, and J) or 0.0031% (lanes B, E, H, and K) or 0.0016% (lanes C, F, I, and L) Triquart Super. The arrows show the sizes of the *plcA-prfA* (2.1-kb) and *prfA* (0.8- and 0.9-kb) transcripts. The results are representative of two independent experiments.

in *L. monocytogenes* EGD grown with both concentrations of Incimaxx DES.

Upregulation of *prfA* and *inlA* in *L. monocytogenes* EGD grown in BHI with Triquart Super was seen on the transcript level with the Northern blot analysis (Fig. 5 and data not shown). The monocistronic *prfA* transcripts were upregulated after 15, 30, and 60 min of growth with 0.0031% Triquart Super (lanes B, E, and H) compared to the control (lane A, D, and G). Growth with 0.0016% Triquart Super also upregulated *prfA* after 60 min. The transcript at 2.1 kb was only weakly expressed, since *L. monocytogenes* was grown in BHI broth without addition of charcoal. Only a weakly monocistronic *inlA* transcript was detected. However induction could be seen after 30 min of growth with 0.0031% Triquart Super (data not shown).

Together, the Northern blot analyses confirmed the observations obtained with the agar assay.

DISCUSSION

In the present study, we have developed an agar-based assay based on an existing assay intended to isolate fungal compounds that reduce virulence gene expression in *S. aureus* (Nielsen et al., unpublished data). Similarly, our assay can be used to study how different compounds affect virulence gene expression in *L. monocytogenes*. In the screening assay, the expression is determined as β-galactosidase activity of virulence gene promoters fused to a *lacZ* gene. However, this relates very well to the transcript level as confirmed by Northern blot analyses. The assay has high sensitivity, since it detects differences in expression on the transcript level, but it is not quantitative. In the present study, we used the assay to determine the effects of different disinfectants routinely used in the food industry on the gene expression of four virulence genes, but the assay could also be used for screening of other compounds and their influence on other (virulence) genes in *L. monocytogenes*.

In general, disinfectants with the same active ingredients had similar effects on virulence gene expression. Peroxy and chlorine compounds reduced the virulence gene expression, and QAC induced the expression of the virulence. Ryan et al. (42) found that two QAC, benzalkoniumchloride (BC) and cetylpyridinium chloride (CPC), at sublethal concentrations, induced expression of *sigB* in *L. monocytogenes* as measured by reverse transcriptase PCR; hence, QAC may affect a number of bacterial behaviors. No housekeeping genes were included

in our study. However, we find it unlikely that QAC induce the expression of all genes in *L. monocytogenes*, as BC and CPC did not induce 16S RNA in the study by Ryan et al. (42). The difference in the effect on gene expression is not due to differences in pH *per se*, since disinfectants with peroxy compounds have a low pH, whereas the disinfectants with chlorine and QAC compounds generally have a high pH. Others have determined the effect of subinhibitory concentrations of commercial disinfectants on virulence in other pathogens. Disinfectants including QAC reduced virulence factors in *Pseudomonas aeruginosa* and *Streptococcus agalactiae* when measured on the phenotypic level (19, 20, 30), benzyl alcohol induced expression of an operon involved in biofilm formation in *Staphylococcus epidermidis*, and peracetic acid induced some and downregulated other virulence-related genes in *S. aureus* when studied by transcriptomics (6, 36).

PrfA is transcribed both as monocistronic *prfA* from its own two promoters and as a *prfA-plcA* bicistron from p*plcA* (4, 16, 32). Basal amounts of PrfA are made from the monocistronic *prfA* transcripts, and these stimulate the transcription of the *prfA-plcA* bicistron. In our Northern blot analysis, no transcript was detected at 2.1 kb in trials with Incimaxx DES and when *L. monocytogenes* EGD was grown in BHI, which could be due to a too-low basal amount of monocistronic *prfA*. The increase caused by Triquart Super was not sufficient to induce the transcription of the *prfA-plcA* bicistron. Interestingly, the effect on promoter activity and transcription level indicates that the disinfectants, even at levels not inhibiting growth, interact with the transcription process directly or indirectly. This could be through interaction with the promoter region or the RNA polymerase. The disinfectant also could act in the allosteric transition of PrfA (e.g., by interacting with the so-far-unidentified cofactor) that is necessary for the protein to stimulate the virulence promoters (41, 48, 49).

It is well known that PrfA regulates the expression of several virulence genes in *L. monocytogenes*, including *hly*, *inlA*, *plcA*, and *prfA*. However, activation by PrfA is more efficient at promoters which posses a perfectly symmetrical PrfA box, such as p*plcA* or p*hly*, than at promoters which have substitutions, such as p*inlAB* (44). Also, some virulence genes have more than one promoter. *plcA* is transcribed from one PrfA-dependent promoter, and *hly* and *inlA* are transcribed from three and four promoters, respectively. Two *hly* promoters are PrfA dependent and one is PrfA independent, whereas only one of the *inlA* promoters is PrfA dependent (9, 10, 28, 33). This might lead to the different dependency on PrfA seen in this study, where only p*inlA* was sufficiently induced during growth in the Δ*prfA* strain to produce a visible amount of β-galactosidase. In the screening assay, the presence of PrfA did not visibly affect the effects of the different disinfectants on p*inlA*. This indicates that the modulation of *inlA* expression by disinfectants is not dependent on PrfA concentration. However, an interaction of PrfA on p*inlA* cannot be excluded by this assay.

To our knowledge, this is the first study showing that at sublethal concentrations disinfectants routinely used in the food industry affect virulence gene expression in the human pathogen *L. monocytogenes* and that the effect depends on the active component in the disinfectant. From a practical perspective, the study underlines that disinfectants should be used at the lethal concentrations recommended by the manufacturers based on data obtained according to accepted disinfectant test standards. Further studies are necessary to elucidate whether these differences have biological importance. One may hypothesize that sublethal concentrations of disinfectants could affect the stress response or virulence of the bacteria. This would be highly relevant in risk analysis of the use of disinfectants, especially in settings where the route of the pathogen from disinfectant exposure to the host is shorter, as it is in food processing settings.

Recently concerns have been expressed about the potential effect of biocide exposure on development of resistance to disinfectants or antibiotics. Resistance to disinfectants is believed to be a relatively rare event because most disinfectants are often complexes of antimicrobials that inactivate multiple cell targets (31). However, sublethal concentrations of disinfectants can select for antibiotic resistance. In *Salmonella enterica* serovar Typhimurium, a QAC showed the highest selectivity for variants with reduced susceptibility to different antibiotics (23). This variant had increased level of *acrB*, a marker for efflux, leading to decreased sensitivity to antibiotics. Also, induction of efflux pumps caused by biocides has been detected in other pathogens (14, 21, 43). Similarly, one may speculate that the increase in virulence gene expression detected with some disinfectants in the present study could be generalized to more genes in *L. monocytogenes* and thereby cause an increase in antibiotic resistance in this bacterium.

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