Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry

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ABSTRACT

The antibacterial effect of disinfectants is crucial for the control of *Listeria monocytogenes* in food processing environments. Tolerance of *L. monocytogenes* to sublethal levels of disinfectants based on quaternary ammonium compounds (QAC) is conferred by the resistance determinants *qacH* and *bcrABC*. The presence and distribution of these genes have been anticipated to have a role in the survival and growth of *L. monocytogenes* in food processing environments where QAC based disinfectants are in common use. In this study, a panel of 680 *L. monocytogenes* from nine Norwegian meat- and salmon processing plants were grouped into 36 MLVA profiles. The presence of *qacH* and *bcrABC* was determined in 101 isolates from the 26 most common MLVA profiles. Five MLVA profiles contained *qacH* and two contained *bcrABC*. Isolates with *qacH* and *bcrABC* showed increased tolerance to the QAC Benzalkonium chloride (BC), with minimal inhibitory concentrations (MICs) of 5–12, 10–13 and <5 ppm for strains with *qacH* (two allele variants observed), *bcrABC*, and neither gene, respectively. Isolates with *qacH* or *bcrABC* were not more tolerant to BC in bactericidal tests in suspension or in biofilms compared with isolates lacking the genes. Water residue samples collected from surfaces in meat processing plants after QAC disinfection had bactericidal effect against *L. monocytogenes* when the sample BC levels were high (>100 ppm). A sample with lower BC concentrations (14 ppm of chain length C-12 and 2.7 ppm of chain length C-14) inhibited growth of *L. monocytogenes* not containing *bcrABC* or *qacH*, compared to strains with these genes.

The study has shown that *L. monocytogenes* harbouring the QAC resistance genes *qacH* and *bcrABC* are prevalent in the food industry and that residuals of QAC may be present in concentrations after sanitation in the industry that result in a growth advantage for bacteria with such resistance genes.

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1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes the severe disease listeriosis (Swaminathan and Gerner-Smidt, 2007; Warriner and Namvar, 2009). Although the prevalence of listeriosis is low (0.52 cases per 100,000 population in the EU in 2014), the severity of the disease makes it one of the most important foodborne pathogens, both economically and with regard to public health (Drevets and Bronze, 2008; European Food Safety Authority, 2015; Ivanek et al., 2004). Although *L. monocytogenes* may survive mild heat treatment at <60 °C, it is relative sensitive to higher temperatures; e.g. D 71 °C < 1 s in foods (Lado and Yousef, 2007). Thus foods that are consumed without further heat treatment, so-called ready-to-eat foods, are the main sources for listeriosis outbreaks. Among outbreaks related to such foods we find cold meat cuts (Gottlieb et al., 2006), smoked fish (Tham et al., 2000), soft cheeses (Fretz et al., 2010; Gaulin et al., 2012; Johnsen

et al., 2010) and recently fresh produce (Laksanalamai et al., 2012). The main contamination route for *L. monocytogenes* is through cross-contamination from equipment/machines to food during processing (Ferreira et al., 2014; Møretrø and Langsrud, 2004). *L. monocytogenes* possesses the ability to establish itself in equipment/production environments, and single strains of *L. monocytogenes* have been found to reoccur in production environments over periods of years (Carpentier and Cerf, 2011; Ferreira et al., 2014; Møretrø and Langsrud, 2004). The bacteria persist in environmental niches like cracks, inside equipment, protected from cleaning and disinfection routines. The mechanisms why certain strains persist in food processing plants are not well understood (Ferreira et al., 2014). Some studies have found that persistent strains have increased biofilm formation (Borucki et al., 2003; Lunden et al., 2000; Norwood and Gilmour, 1999) or tolerance to stress conditions like disinfection (Aase et al., 2000), but other studies found no association between persistence and these specific phenotypic characteristics (Djordjevic et al., 2002; Harvey et al., 2007; Heir et al., 2004; Holah et al., 2002). Carpentier and Cerf (2011) suggest that environmental niches are more important for persistence than differences between strains.

Quaternary ammonium compounds (QAC) are cationic membrane active antibacterial agents widely used in disinfectants in health care, agriculture, home and the food industry (Gerba, 2015; Tezel and Pavlostathis, 2015). Benzalkonium chloride (BC) is a commonly used type of QAC, which typical contains a mixture of molecules with alkyl chain lengths of C12-C16. The tolerance of *L. monocytogenes* to BC varies between strains (Aase et al., 2000; Heir et al., 2004; Mereghetti et al., 2000), and *L. monocytogenes* have been shown to possess at least two genetic determinants that enhance their tolerance towards QAC, *qacH* (Müller et al., 2013; Müller et al., 2014) and *bcrABC* (Dutta et al., 2013). QacH, BcrB, and BcrC are transporters belonging to the small multidrug resistance (SMR) protein family. Recently, it was shown that a prevalent persistent sequence type of *L. monocytogenes* in Europe is highly conserved and harbors the transposon, Tn*6188*, responsible for increased tolerance against QAC through *qacH* and it was suggested that this contributes to survival and persistence (Müller et al., 2014). The tolerance level for *qacH* or *bcrABC*-positive strains is considerably lower than the normal user concentration of QAC (Tezel and Pavlostathis, 2015), and the relevance of these genes for growth and survival of bacteria in the food industry has been questioned (Gerba, 2015; Kastbjerg and Gram, 2012).

In a normal sanitation cycle process in the food industry a cleaning agent is applied and rinsed off with water before the disinfectant is applied. After a certain exposure time (usually minutes) the disinfectant is rinsed off with water. If the rinsing is insufficient, residues of the disinfectant may remain on the surface. The objectives of this study were to investigate the prevalence of the resistance genes *qacH* and *bcrABC* among common *L. monocytogenes* MLVA types in the Norwegian meat and salmon processing industry and to evaluate whether these genes may have any practical impact on *Listeria* growth, survival and protection against QAC based disinfectants in food industry settings. It was evaluated whether residues of QAC remains after disinfection and how growth and survival of *L. monocytogenes* with various QAC tolerance was affected under such conditions. Also potential protective effects of *qacH* or *bcrABC* during bactericidal tests against BC in suspension and biofilms were investigated.

2. Materials and methods

2.1. Sampling of L. monocytogenes in salmon- and meat processing plants

Four salmon processing facilities and five RTE meat production facilities (Table 1) were sampled 3–5 times within a period of three years. A harmonized sampling plan including floor-associated points (drains, floors, wheels and shoe washers (if present)) and contact points (conveyor belts, slicers (if present)) was used. Vacuum systems in the gutting process of salmon were included for all salmon plants. Additional sampling points were plant-specific sites identified as *Listeria*-positive

Table 1
Overview over visited production sites, products and disinfection agents.

Meat (M) and Salmon (S) processing plants	Main products	Main disinfectant used in sampling period
M1	Sausages	QAC
M2	Poultry RTE-products	QAC
M3	Cold cuts	QAC/GA
M4	Sausages, cold cuts	PA
M5	Slicing of cold cuts, cured meat, others	QAC and HC (weekly alternation)
S1	Smoked salmon and fresh salmon fillet	PA (HC once a week)
S2	Gutted salmon	PA (fogging)
S3	Smoked salmon and fresh salmon fillet	QAC
S4	Fresh salmon fillet	Chlorine dioxide

Abbreviations: QAC; Quaternary ammonium compounds, PA; Peracetic acid, HC; Hypochlorite, GA; glutaraldehyde.

in the regular monitoring program or suspected to be *Listeria* niches (rubber sealings under doors, integrated floor weights, storage crates, slicers, control panels, wielding joints, rubber/metal transitions, damaged floor surfaces and potential water accumulation sites). Two sets of sampling were carried out, the first after sanitation, the second during production, at least 2 h after production started. A total number of 50–80 samples were taken at each plant. Some sites were unavailable for sampling during production (slicers, vacuum systems, insides of equipment) and were hence sampled before production only.

Samples were taken using neutralizing sampling cloths (SodiBox, Nevez, France) and where possible, an area of approximately 30 × 30 cm was sampled. Cloths were stored at 4 °C until analysis, not >24 h after sampling. The sampling cloths were analyzed for qualitative detection of *Listeria monocytogenes* according to NMKL method no. 136 (NMKL, 2007). In short, the cloths were added to 100 ml 1/2 Fraser broth with selective supplement (Oxoid, Basingstoke, UK) for pre-enrichment for 24 h at 30 °C. One ml of the pre-enrichment broth was transferred to Fraser broth with selective supplement (10 ml) (Oxoid) for secondary enrichment at 37 °C for 48 h. Cultures from positive enrichment broths were plated on RAPID'L.mono agar (RLmA; Biorad). Presumptive *L. monocytogenes* colonies from enrichment and selective plating were confirmed using a *L. monocytogenes* specific PCR (Wesley et al., 2002).

2.2. Multiple locus variable number tandem-repeats analysis (MLVA)

All isolates, a total of 313 from meat production sites and 367 from salmon processing sites, were typed by MLVA by The Norwegian Institute for Public Health according to the method described by Lindstedt et al. (2008). Briefly, the size of five selected repetitive DNA units (Variable Number Tandem Repeats, VNTR) was determined, and each isolate was given a five-digit code based on the number of repetitive units (one digit for each tandem repeat unit).

2.3. Multilocus sequence typing (MLST)

MLST was performed as described by Ragon et al. (2008). Alleles and sequence types for MLST were compared with those available at http://bigsdb.web.pasteur.fr/listeria.

2.4. Prevalence of qacH and bcrABC

The prevalence of *qacH* and *bcrABC* was assessed for 101 selected isolates. For frequently isolated MLVA profiles (those with at least ten isolates), two isolates were selected from each producer (wherever possible), preferably isolates recovered after sanitation. For less frequently isolated MLVA profiles (with two to nine isolates recovered), one isolate from each producer was included, when possible isolated after sanitation. Isolates were cultured on Tryptic Soy agar (TSA) plates for 24 h at 37 °C and one colony of each isolate was transferred to one well of a 96 well PCR plate. The plate was microwaved for 1 min to lyse the cells. The PCR mix added to each well contained 12.5 µl 2× Qiagen Multiplex PCR Master Mix (Qiagen), primers (0.2 µM final concentration) and water (to a final volume of 25 µl). The following primers were used: BcF and BcR (Elhanafi et al., 2010) for amplification of *bcrABC* or qacH fwd and qacH rev (Müller et al., 2013) for amplification of *qacH*. The cycling conditions for *qacH* were as follows: 15 min of initiating at 95 °C, 30 cycles of 30 s of denaturation at 94 °C, 90 s of annealing at 56 °C and 30 s of elongation at 72 °C followed by a final elongation step of 10 min at 72 °C. For *bcrABC*, the annealing temperature was 60 °C and the elongation step was 90 s. The presence of *qacH*/*bcrABC* was checked by agarose gel electrophoresis. The *qacH* PCR products from selected isolates were sequenced using the same primers as were used in PCR, thus obtaining the sequence of the central 329 bp of the 372 bp *qacH* gene.

2.5. Phylogenetic tree construction

The *L. monocytogenes* genomes available in GenBank as of 19. Nov. 2015 were downloaded and MLST typed in silico as described in Kwong et al. (2016). The 352 genomes for which complete MLST profiles were obtained were further analyzed for the presence of *qacH* and *bcrABC* using BLASTN. For the 72 MLST sequence types (ST) present in either the set of downloaded genomes or in the isolates collected in the current study, the concatenated sequences of the seven MLST alleles were aligned. A neighbor joining phylogenetic tree was then constructed using CLC Main Workbench 7.5 (CLCbio). Genetic distances were estimated using the Jukes-Cantor model.

2.6. Genome sequencing and assembly

For purification of genomic DNA, cells were lysed using Lysing Matrix B and a FastPrep instrument (both MP Biomedicals, Illkirch-Graffenstaden, France), and DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Limburg, Netherlands). Libraries for genome sequencing were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, USA) and sequenced using paired-end 2×300 bp reads on a MiSeq instrument (Illumina). Reads were assembled de novo with SPAdes v3.0.0 (Bankevich et al., 2012).

2.7. Survival and growth during exposure to Benzalkonium chloride (BC)

For all growth and survival experiments, a fresh starting culture of the strains was prepared by cultivation in 5 ml Tryptic Soy Broth (TSB, Oxoid) for 24 h at 30 °C and 200 rpm resulting in a concentration of ~ 2×10^9 cfu ml^{-1}. All experiments were performed in triplicate.

2.7.1. Minimum inhibitory concentration (MIC) assays

The minimum inhibitory concentration (MIC) of BC (Sigma) was determined for 101 selected *L. monocytogenes* isolates. BC was diluted in TSB supplemented with 0.6% yeast extract (TSB + YE), and 180 μl was added to the wells of a 96 well microtiter plate (Fisher Scientific, Oslo, Norway). In initial experiments, the final BC concentrations tested were 0, 0.5, 1, 2, 4, 6, 8 and 10 ppm. Isolates with initial MIC values of 4 or lower were then tested at 1–4 ppm BC (0.5 ppm intervals), while isolates with initial MIC values of 6 or higher were tested at 4-14 ppm BC (1 ppm intervals). The overnight culture was diluted 1:100 in TSB + YE, and 20 μl were added to the wells, resulting in a starting concentration in the wells of ~2×10^6 cfu ml^{-1}. Plates were incubated at 30 °C for two days and optical density (600 nm) was measured (Multiskan RC, Labsystems). A cut-off value for growth was set at 0.1 after subtraction of the control sample.

2.7.2. Bactericidal suspension test

The culture was diluted 1:10 in peptone water (1 g l^{-1} peptone (Oxoid), 0.85% NaCl, pH 7.2) and 0.5 ml of the diluted culture was added directly to 4.5 ml of dH$_2$O (control) or BC (10 ppm final concentration) resulting in a final cell concentration of 10^7 CFU ml^{-1}. After 5 min, 0.5 ml of the solution was transferred to Dey Engley neutralizing broth (D/E, Difco) and plated on TSA. Plates were incubated at 37 °C for 24 h.

2.7.3. Bactericidal biofilm test

Overnight cultures were diluted 1:1000 in TSB + YE. 5 ml of this diluted culture was added to the wells of a 6-well Tissue Culture plate (Fisher Scientific) containing a steel coupon (2×2 cm, AISI 304, 2B, Norsk Stål, Nesbru, Norway) in each well. The plates were incubated at 30 °C for 3 h before the suspension was carefully pipetted off. 5 ml of dH$_2$O were carefully added to the coupons and pipetted off again to remove unattached cells. The coupons were transferred to fresh plates, 3 ml of TSB + YE were added and the plates were incubated at 30 °C for 48 h. After incubation, the remaining suspension was removed by

pipetting and coupons rinsed as before by adding and removing 3 ml dH$_2$O. Coupons were then transferred to glass tubes containing 6 ml of dH$_2$O (control) or BC (50 ppm). After 5 min, coupons were transferred to glass tubes containing 6 ml of D/E and sonicated for 10 min at 40 kHz (Bransonic 3510, Branson Ultrasonic B. V., Soest, The Netherlands). The suspension was plated on TSA and incubated at 37 °C for 24 h.

2.8. Residuals of benzalkonium chloride after disinfection

2.8.1. Sampling of residual water after rinsing in two meat processing plants

Samples of residual water on surfaces in two meat processing plants after the daily sanitation cycle was collected to test for residuals of disinfectants. Twelve and fifteen water samples were collected from two meat producers (M1 and M2), respectively within 1 h after ended sanitation cycle. The samples were collected at sites where water was observed to accumulate after sanitation (floors, conveyor belts, machines/equipment). Ten to 15 ml of water was transferred to tubes which were frozen at −20 °C until further analysis. The sanitation cycle consisted of rinsing with potable water, applying of foaming cleaning agent, rinsing with potable water, applying of foaming disinfectant and rinsing with potable water. Both plants used DesQA (Ako Kjemi, Lillehammer, Norway), with BC as active component for disinfection.

2.8.2. Growth rate at low BC concentrations

The aim was to compare the growth of *L. monocytogenes* isolates with or without the QAC resistance genes *bcrABC* or *qacH* at BC concentrations and conditions that may be present after sanitation. The five strains tested included one *bcrABC* positive (MF4624[bcrABC]), one isolate with *qacH* with cysteine in position 42 (MF5380[QacH42cys]), one isolate with *qacH* with serine in position 42 (MF5634[QacH42ser]) and two control isolates lacking both *bcrABC* and *qacH* (MF5376[ctrl] and MF4993[ctrl]). Low concentrations of nutrients as well as 12 °C were used as this is in line with the conditions in the processing plants after sanitation. Growth was tested for dilutions of BC and the commercial BC-containing disinfectant DesQA. An overnight culture was diluted 1:100 in TSB + 0.6% YE and 20 μl of the diluted culture was added to the wells of 100-well plates (Oy Growth Curves Ab Ltd., Helsinki, Finland) with 180 μl of BC or DesQA diluted in 1/10 TSB + 0.06% YE to suitable concentrations. The plates were incubated at 12 °C and the optical density was measured automatically every 30 min (with 10 s shaking before each measurement) for six days using a Bioscreen FP-1100-C (Oy Growth Curves Ab Ltd).

Growth was also tested for *L. monocytogenes* in presence of the 27 water residue samples collected after sanitation, as described above by replacing the BC solutions with the water samples. The water samples were filter sterilized (Millex – GS 0.22 μm, Merck Millipore LTD) before the experiment. The wells contained 20 μl bacterial culture prepared as described above, 20 μl TSB + 0.6% YE and 160 μl of the water sample. Bacterial growth was observed and measured as described above.

2.8.3. Benzalkonium chloride concentrations in water residues after sanitation

A total of three and five water samples collected after sanitation from food processing plants M1 and M2, respectively, were analyzed for the presence of BC. The samples were selected based on their effect against *L. monocytogenes* in the experiments described above. Three of the analyzed samples inhibited *L. monocytogenes*, while the last five water samples had no effect on the growth of *L. monocytogenes*. The analyses were purchased from an analytical laboratory at a governmental research institute (NIBIO - Norwegian Institute of Bioeconomy Research, Plant Health and Biotechnology, Pesticide Chemistry, Ås, Norway) which used QuEChERS extraction followed by a LC-MS/MS analysis for quaternary ammonium compounds (Anastassiades et al., 2003; European Reference Laboratories for Residues of Pesticides, 2014).

Table 2

L. monocytogenes MLVA profiles isolated at least twice from the sampled meat and salmon poduction plants. Numbers in italics indicate numbers of isolates found in the various production plants. MLST profiles, presence of *qacH* and *bcrABC* genes, and MIC (BC) are shown for selected isolates.

MLVA no.	MLVA	Meat plants					Salmon plants				Characteristics of selected isolates				
		M1	M2	M3	M4	M5	S1	S2	S3	S4	No. isolates tested	ST[a]	*qacH*	*bcrABC*	MIC (BC) [ppm]
1	58-13-12-6						*3*				1	177	−	−	4
2	5-8-14-10-6						*2*	*9*	*5*		4	14	−	−	2–4
3	5-8-15-10-6						*12*	*17*	*31*		9	14	−	−	2–4
4	5-8-16-10-6						*1*	*13*			3	14	−	−	2–4
5	6-0-14-10-6				*1*		*2*				1	121	+	−	11
6	6-7-14-10-6			*1*	*9*		*59*	*1*		*14*	12	121	+	−	10–12
7	6-7-15-10-6						*4*				1	121	+	−	11
8	6-8-14-18-6						*4*	*2*		*19*	7	31	−	−	2–4
9	6-9-14-16-6				*2*						1	451	−	−	4
10	6-9-18-10-6	*1*			*7*	*1*					3	19[b]	−	−	2–4
11	6-9-18-16-6		*3*			*3*	*48*		*1*		12	8	−	−	2–4
12	6-9-19-10-6		*2*		*12*						2	394	−	−	2–4
13	6-9-26-16-6							*3*			1	551	−	−	3
14	6-10-2-22-6	*4*									2	91	−	−	2–4
15	6-10-5-16-6						*2*			*31*	4	20	−	−	2–4
16	6-11-15-18-6	*39*			*67*	*4*					8	9	+[d]	−	5–6[d]
17	7-7-10-10-6		*12*		*2*		*55*	*7*			10	7[c]	−	−	2–4
18	7-7-11-10-6						*1*	*1*			2	995	−	−	2–3
19	7-7-13-6-10		*2*								1	2	+	−	8
20	7-8-17-18-6						*2*				1	18	−	−	3
21	7-10-15-18-6	*26*									4	9	−	+	10–11
22	7-11-15-18-6	*5*			*78*						4	9	−	+	10–13
23	8-8-17-16-6				*5*		*1*				3	18	−	−	2–3
24	8-8-17-18-6						*4*				2	18	−	−	3–5
25	8-8-17-19-6				*4*		*4*				2	18	−	−	2–4
26	8-8-17-21-6	*3*									1	18	−	−	3

[a] ST numbers refer to Institut Pasteur MLST database (http://bigsdb.web.pasteur.fr/listeria/listeria.html).

[b] One of three isolates had MLST profile 394.

[c] One of eight isolates had MLST profile 732.

[d] One of eight isolates *qacH* negative, MIC 4 ppm.

2.8.4. Statistics

The differences in susceptibility between isolates with and without *qacH* or *bcrABC* was tested by 2-sample t-tests in Minitab (Minitab, version 17.3.1).

3. Results

3.1. Strain diversity

A total of 313 *L. monocytogenes* isolates from the meat industry and 367 isolates from the salmon industry were typed using MLVA and 36 distinct MLVA profiles were found. Eleven of these were isolated frequently (ten isolates or more), while 10 profiles were isolated only once. All MLVA profiles isolated at least twice are listed in Table 2.

Ninety of these isolates were subjected to MLST typing (Table 2). This was done to confirm the ability of MLVA to separate *L. monocytogenes* isolated and to facilitate comparison to other strains in publications using MLST for identification. Results showed that MLVA yielded a higher resolution than MLST and the 26 MLVA profiles tested were distributed to only 16 MLST sequence types (STs). Several STs comprised more than one MLVA profile while isolates with identical MLVA profiles all had identical STs with two exceptions: Among the seven MLST typed isolates with MLVA profile 17 (Table 2), six isolates were ST7 and one was ST732, and of the three MLST typed isolates with MLVA profile 10, two isolates were ST19, while one was ST394.
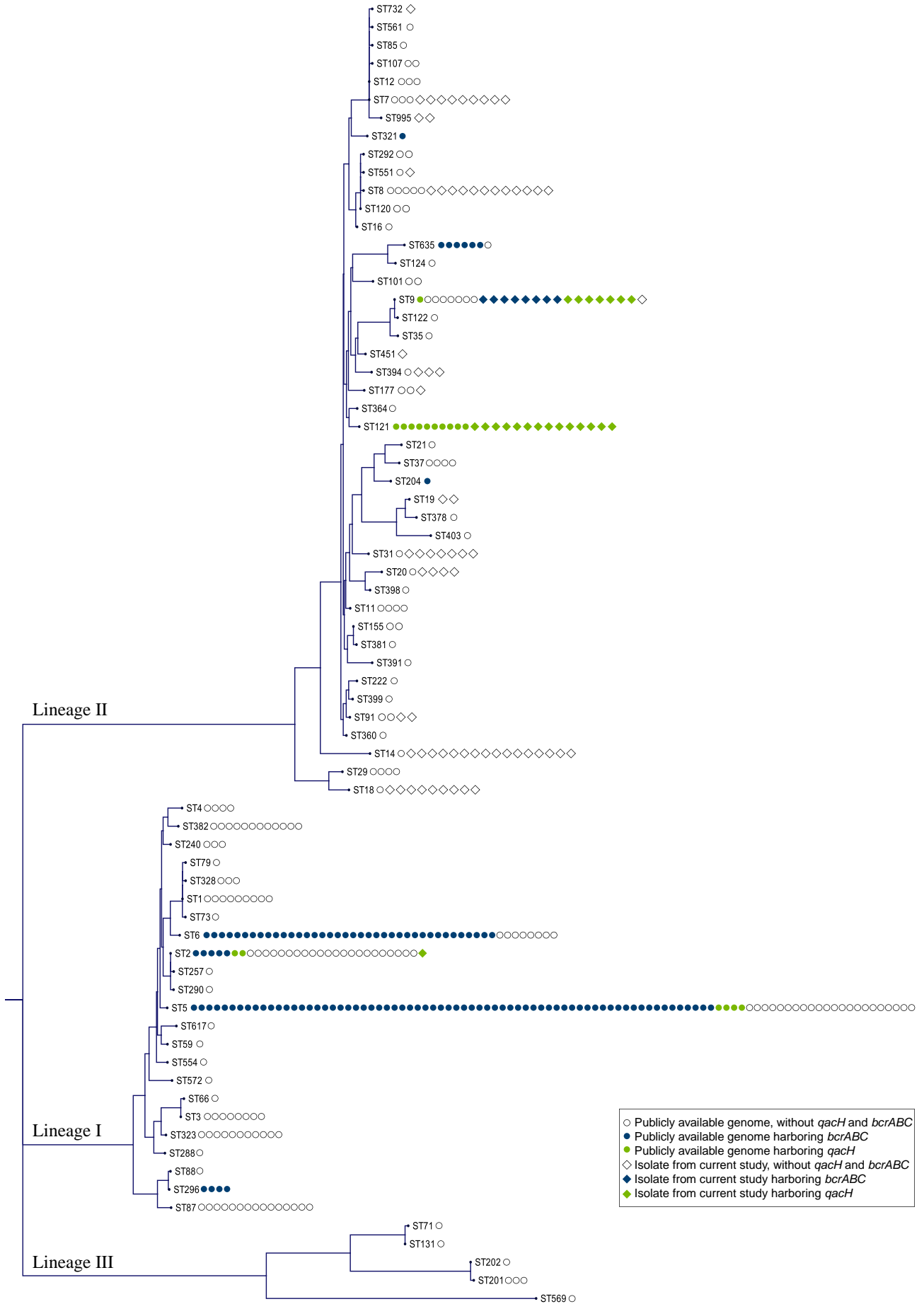
3.2. Presence of qacH and bcrABC

A total of 101 isolates were selected for further characterization regarding sensitivity to BC and genetic determinants for BC tolerance (Table 2). The isolates were subjected to PCR to determine whether they harboured the *qacH* gene or the *bcrABC* operon. A total of 23 isolates (22%) were positive for *qacH*, while 8 isolates (8%) harboured *bcrABC*. None of the isolates contained both *qacH* and *bcrABC*. Isolates with identical MLVA profiles had identical distributions of the two genes *qacH* and *bcrABC*, with one exception: Of the eight isolates with MLVA profile 16, one strain was *qacH* negative. *qacH* genes were found in five MLVA profiles (5, 6, 7, 16, and 19) corresponding to MLST types ST121, ST9, and ST2, while *bcrABC* was detected in two MLVA profiles (21 and 22), both ST9 (Table 2).

To examine whether the correlation of *qacH* and *bcrABC* genes with phylogenetic group was found also in other *L. monocytogenes* strains, publicly available *L. monocytogenes* genome sequences were typed using in silico MLST and analyzed for the presence of *qacH* and *bcrABC*. The results for both the 101 selected isolates from the current study and the 352 publicly available genomes are presented in Fig. 1. The *qacH* gene was found in four of the 72 represented MLST types. These were the highly sampled ST2 and ST5 from lineage I, and ST9 and ST121 from lineage II.

The *bcrABC* operon was identified in a total of eight MLST STs, namely in ST2, ST5, ST6, and ST296 of lineage I, and in ST9, ST204, ST321, and ST635 of lineage II. In the current study, the only identified *bcrABC*-positive isolates were from the ST9 group (Fig. 1).

Fig. 1. Distribution of *qacH* and *bcrABC* genes within the *L. monocytogenes* population. A phylogenetic tree obtained from the concatenated nucleotide sequences of 72 MLST STs was constructed as described (Materials and methods). The numbering in the figure refers to the ST. The three genetic lineages (I to III) are marked. Each symbol following the ST designation on each branch of the tree represents one isolate or genome. Circles indicate publicly available genomes, while diamonds indicate isolates from the current study. Blue symbols indicate the presence of *bcrABC*, while green symbols indicate the presence of *qacH*.

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                                                                    20                        40
                                                                     |                         |
ST121, MLVA profiles 5 and 6    MS Y LY LA LA I V GE I I GS S L LK AS EG FS KL FP T I GV I I AF VV S  42
ST9 and ST2, MLVA profiles 16 and 19  MS Y LY LA LA I V GE I I GS S L LK AS EG FS KL FP T I GV I I AF VV C  42
ST121, MLVA profile 7           - - - - - - - - L A I V GE I I GS S L LK AS EG FS KL FP T I GV I I AF VV S  35
HPB2262_ST2                     MS Y LY LA LA I V GE I T GS S L LK AS EG FS KL FP T I GV I I AF VV S  42
ST5                             MS Y LY LA LA I V GE I I GS S L LK AS EG FS KL Y PT IG VI I A F VGC  42

                                                                    60                        80
                                                                     |                         |
ST121, MLVA profiles 5 and 6    F F F LS LS LK TI PL NT AYS LWS GL GL VL TT I I S V LI WK EK LN M  84
ST9 and ST2, MLVA profiles 16 and 19  F F F LS LS LK TI PL NT AY AL WA GL GL VL TT I I S V LI WK EK LN M  84
ST121, MLVA profile 7           F F F LS LS LK TI PL NT AY AL WA GL GL VL TT I I S V LI WK EK LN M  77
HPB2262_ST2                     F S FL SL SL KT IP LN TA YA LW AG L G LV LT TI IS VL IWKE KL NM  84
ST5                             F F F LS LS LK TI S LN TA YA LW AG L G LV LT TV IS VL V WK EK LN M  84

                                                                   100                       120
                                                                     |                         |
ST121, MLVA profiles 5 and 6    A S I AG IT LI LA GV VI LN LF GP GH GE PD HK AS ET IT IN E - - E  123
ST9 and ST2, MLVA profiles 16 and 19  A S I AG IT LI LA GV VI LN LF GP GH GE PD HK AS ET IT IN E - - E  123
ST121, MLVA profile 7           A S I AG IT LI I AG VV IL NL FG PG HG EP DH KA SE - - - - - - - - -  109
HPB2262_ST2                     A S I AG IT LI LA GV VI LN LF GP GH GE PD HK AS ET IT IN E - - E  123
ST5                             A S I AG IT LI LV GV VI LN LF GP GH GE SG HE AS ET IT MNE TME  125
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Fig. 2. QacH protein sequence alignment. The QacH protein sequences shown are from the following *L. monocytogenes* isolates, with STs and GenBank accessions indicated in parenthesis: *ST121, MLVA profiles 5 and 6*: QacH from the three MLVA profile 6 (ST121) isolates MF3858, MF5216, and MF5634, and from one MLVA profile 5 (ST121) isolate (MF3908). In addition, the following publicly available ST121 *L. monocytogenes* WGS encode this QacH variant: 6179 (HG813249), Lm_1880 (KK074110), 3253 (JYJO01000006), N53-1 (AXDU01000007), 4423 (CBXR010000008), MOD1_LS889 (JRZA01000002), S2_3 (JWHK01000031), S2_2 (JWHJ01000009), S10_1 (JWHG01000069), and S10_3 (JWHH01000037). *ST9 and ST2, MLVA profiles 16 and 19*: QacH from the four MLVA profile 16 (ST9) isolates MF4545, MF4562, MF5380, and MF5628 from the MLVA profile 19 (ST2) isolate MF4990, and from the following publicly available *L. monocytogenes* WGS from 5KSM (ST9; JYOK01000008) and Lm_1824 (ST2; KK074021). *ST121, MLVA profile 7*: QacH from the MLVA profile 7 (ST121) isolate (MF5095). *HPB2262_ST2*: QacH from the publicly available WGS from ST2 *L. monocytogenes* strain HPB2262 (AATL02000003). *ST5*: QacH from the publicly available ST5 *L. monocytogenes* WGSs from FSL R8-5124 (JOWR01000002), FSL R8-6480 (JOZH01000002), FSL R8-7153 (JPBD01000002), and FSL R8-7478 (JPBO01000001). The sequences for isolates MF3858, MF5216, MF3908, MF5095, MF5380, MF5628, and MF4990 are from sequencing of PCR products and thus only the central 109 amino acids of QacH was obtained for these isolates.

The prevalence of *qacH* in the 352 publicly available sequences was 5% (compared with 22% in our isolates), while 43% of sequenced strains contained *bcrABC* (compared with 8% for our isolates). With the exception of the single isolate from ST2, all examined isolates from the current study belonged to lineage II.

3.3. Exposure of L. monocytogenes to BC in laboratory tests

The 101 *L. monocytogenes* isolates for which presence of genetic determinants for BC tolerance was determined, were subjected to MIC experiments to determine sensitivity to BC. The MICs for the 70 isolates containing neither *qacH* nor *bcrABC* were ≤5 ppm BC while isolates containing *qacH* (23 isolates) and *bcrABC* (8 isolates) had MIC values of 5–12 ppm BC and 10–13 ppm BC, respectively (Table 2). The *qacH* positive isolates could be further subgrouped: The isolates with MLVA profile 16 (MLST ST9) showed MIC values of 5–6 ppm BC, the isolates of MLVA profiles 5–7 (ST121) showed MIC values of 10–12 ppm BC, while the

single tested isolate of MLVA profile 19 (ST2) showed a MIC of 8 ppm BC.

To find the cause for the differences in sensitivity to BC between groups of *qacH* positive isolates, *qacH* sequence comparisons were carried out for selected strains. For three isolates; one of the isolates with MLVA profile 6, and two of the isolates with MLVA profile 16, the *qacH* sequences were obtained through whole genome sequencing (WGS). In addition, the *qacH* PCR products from isolates belonging to six different MLVA profiles were sequenced, thus obtaining the sequence encoding the central 109 amino acids (aa) of QacH for these isolates. The alignment presented in Fig. 2 also includes the QacH sequences found from publicly available *L. monocytogenes* genome sequences. Four single nucleotide differences were found in the *qacH* genes of the isolates tested for BC sensitivity in the current study. These resulted in the following amino acid differences: Cys/Ser at aa 42, Ser/Ala at aa 60 and 63, and Ile/Leu at aa 94 (Fig. 2). Within the group of tested ST121 isolates, which all had MICs of 10–12 ppm BC, the protein encoded by *qacH* in the isolate from MLVA profile 7 differed from the isolates from MLVA profiles 5 and 6 in the last three variant sites, but all harboured [42]Ser. In contrast, the isolates from MLVA profiles 12 (ST9) and 19 (ST2), which had MICs of 5–8 ppm BC, had identical *qacH* genes, encoding [42]Cys. Thus the isolates with QacH variants harbouring [42]Ser had higher tolerance towards BC than those with Cys in this position. Examination of the genome sequences obtained for the MLVA profile 6 isolate (MF5634) and the two isolates with MLVA profile 16 (MF4545 and MF4562) showed that the *qacH* gene resided on the 5 kb transposon Tn*6188* inserted into the *radC* gene, exactly as described for *L. monocytogenes* strains 4423 and 6179 by Müller et al. (2013). The MLVA profile 6 isolate differed from the MLVA profile 16 isolates in the first three mentioned variant sites of *qacH*, but no further differences were found elsewhere in the Tn*6188* transposon.

Individual cultures of nine *L. monocytogenes* isolates were exposed to 10 ppm BC in suspensions and 50 ppm BC in biofilms. The isolates were selected based on their distribution of *qacH* and *bcrABC* and included two *bcrABC* positive, two *qacH*[42cys] positive, two *qacH*[42ser] positive and three isolates negative for both *bcrABC* and *qacH*. The bacterial reductions of the different isolates were 0.6–2.2 log units both in suspension
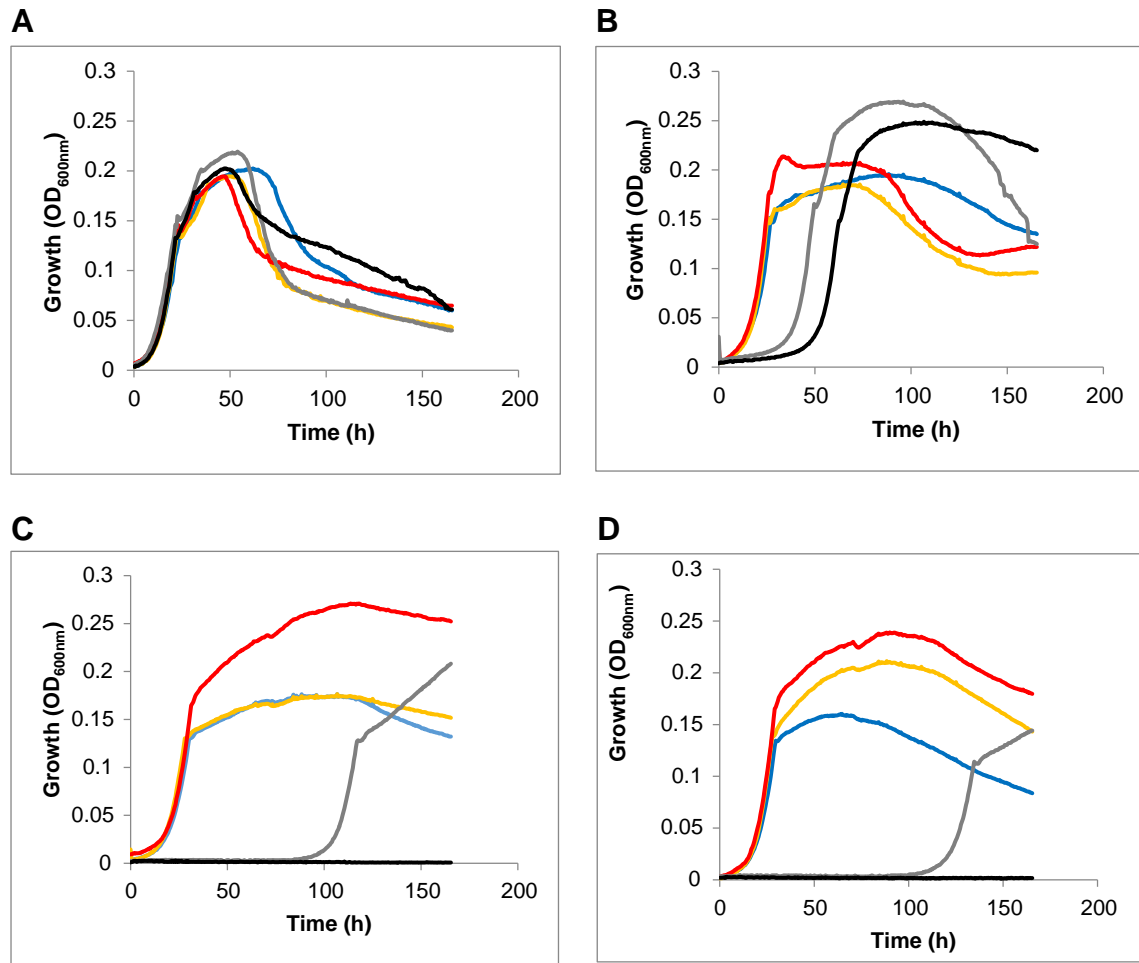
Table 3
Concentration of Benzalkonium chloride (BC) and effect on *L. monocytogenes* of water samples collected from meat processing plants after sanitation.

Sample no.	BC-12[a]	BC-14[a]	Effect on *L. monocytogenes*
M1-9	2.5[b]	0.35	—[c]
M1-11	2.6	0.90	—
M1-12	6.7	0.85	—
M2-1	0.23	0.16	—
M2-2	0.13	0.06	—
M2-9	341	124	+[d]
M2-11	14	2.7	+[e]
M2-14	196	51	+[d]

[a] Indicates result from analysis for BC with chain length of C-12 and C-14.

[b] ppm.

[c] No effect on growth of *L. monocytogenes*. All water samples were diluted 25% in the growth test.

[d] Bactericidal effect (>3 log reduction) on *L. monocytogenes* MF4624[bcrABC], MF5380-[qacH42cys], MF5634[qacH42ser], MF5376[ctrl] and MF4993[ctrl]. Ctrl = control isolate without *qacH* or *bcrABC*.

[e] Increased lag phase of growth of *L. monocytogenes* MF5376[ctrl] and MF4993[ctrl].

Fig. 3. Growth (OD600nm, subtracted readings for uninoculated controls) at 12 °C of *L. monocytogenes* strains MF4624 (*bcrABC* positive); blue, MF5380 (*qacH42cys* positive); red, MF5634 (*qacH42ser* positive); orange, MF4993 (*bcrABC* and *qacH* negative); grey and MF5376 (*bcrABC* and *qacH* negative); black. A and B: Water samples M2–1 and M2–11, respectively, collected after sanitation from a meat processing plant. C: 1 ppm benzalkonium chloride. D: 0.001% of the benzalkonium chloride based disinfectant DesQA. All samples contained 1/10 TSB + 0.06% yeast extract as growth medium. The experimental setup resulted in 25% dilution of the water samples. Results presented were representative for 3–4 independent experiments.

and in biofilm. Strains containing *qacH* or *bcrABC* did not have higher survival during exposure to BC in either biofilm or suspension (p > 0.05). Higher survival in suspension did not lead to higher survival in biofilm. As an example, MF4990 (MLVA profile 19) presented the highest survival in suspension, although the lowest survival in biofilm. The viable cell counts in untreated controls were 6.4–6.6 log per ml in suspension and 6.3–7.1 log/cm² in biofilms.

3.4. Residuals of QAC after disinfection in meat processing plants

The effect of growth and survival of five isolates of *L. monocytogenes* exposed to water samples with QAC residues was tested. The water samples were collected after sanitation (including a disinfection step with DesQA, a BC based disinfectant) in two meat processing plants. Three of a total of 15 water samples from processing plant M2 affected growth/survival of *L. monocytogenes*, while none samples from M1 affected growth. Two of the samples from M2 killed all the five isolates (Table 3). In the third sample (M2-11), the lag phase of isolates harbouring *qacH* or *bcrABC* was shorter than the lag phases observed for isolates without these genes (Fig. 3B). These three water samples, in addition to four samples with no effect on *L. monocytogenes* growth were analyzed for BC. BC was detected in all seven water samples (Table 3). The highest concentrations of BC were found in the three water samples with adverse effects against *L. monocytogenes* (Table 3, Fig. 3A,B).

Both meat processing plants M1 and M2 used the BC-containing disinfectant DesQA. The five *L. monocytogenes* isolates applied in the residual water sample testing from plant M1 and M2 were also tested for growth in presence of sublethal concentrations of DesQA and BC. Concentrations of 0.001% DesQA or 1 ppm BC led to increased lag phase or growth inhibition of the *L. monocytogenes* isolates not harbouring *qacH* or *bcrABC,* compared to isolates containing these genes (Fig. 3C,D). In some technical replicates, the isolates grew after a lag phase, while in other replicates no growth occurred during the incubation time of 165 h. For isolate MF4993[ctrl], there was growth after a lag phase of 80–130 h in 25 technical replicates (data from all replicates), and no growth in eight, while for isolate MF5376[ctrl] there was growth after a lag phase in four technical replicates and no growth in 20 technical replicates. It was confirmed that the observed lag phase was due to growth inhibition and not reduction in viability.

When cultures that grew were removed after 165 h incubation and used to inoculate new wells containing medium with 1 ppm BC, growth was observed in the new cultures within 10 h incubation. It was tested if the tolerance acquired after adaptation was stable when culturing the isolates in absence of QAC. In general the previously adapted MF4993 lost some tolerance to BC when cultured without QAC for 10–60 generations, but lag time (40–140 h) was still shorter than for the wild type (no growth in 6 wells) in cultures containing 1 ppm BC. For MF5376 [ctrl], growth after ca 10 h lag phase was observed at 0.001% DesQA for previously adapted isolates cultured without QAC for 10–60 generations,

while the wild type grew after >100 h lag phase. At 2 ppm BC only the isolates with *qacH*/*bcrABC* grew, the strain harbouring *qacH*^{42cys} grew after a 30–100 h lag time. No growth of any strain was observed at 3 ppm BC.

4. Discussion

Numerous studies have demonstrated increased tolerance to QAC among *L. monocytogenes* strains containing the efflux pump genes *qacH* or *bcrABC* (Dutta et al., 2013; Dutta et al., 2014; Elhanafi et al., 2010; Müller et al., 2013; Müller et al., 2014). However, tolerance levels among *L. monocytogenes* containing these genes have been found to be 30–40 ppm QAC (agar-based MIC assays) (Elhanafi et al., 2010; Müller et al., 2014) while user concentrations of QAC in an industrial context are commonly 200–1000 ppm. The question has hence been raised whether this tolerance level has any practical relevance in the food industry (Gerba, 2015; Kastbjerg and Gram, 2012). Results from this study indicate that there are indeed conditions present where *L. monocytogenes* with *qacH*/*bcrABC* genes may have a growth advantage in the food industry, namely when QAC containing disinfectants are not rinsed off properly after sanitation.

Analysis of water samples showed that residues of QAC were present in water remaining on surfaces after sanitation in two meat processing plants. Although increased growth of *L. monocytogenes* strains with *qacH*/*bcrABC* was only shown in one of the industrial water samples, the wide range of QAC residue concentrations in the analyzed samples indicates that niches with QAC concentrations where *qacH*/*bcrABC* will result in a growth advantage of *L. monocytogenes,* may occur. The growth advantage was demonstrated at temperatures relevant for food industries (12 °C), and in diluted growth media, which is relevant since most nutrients will be removed by the sanitation step. In many cases bad hygienic design or a tight schedule will not allow for water remaining after sanitation to dry up before start of production, resulting in potential niches for growth of QAC-tolerant *L. monocytogenes.* Other genes protecting against QAC may occur, but their importance seems to be limited among the isolates tested in this study, as all isolates without both *bcrABC* and *qacH* were sensitive towards QAC. After the experimental part of the present study had been performed, a novel efflux pump gene (*emrE*) was reported in *L. monocytogenes* isolates from Canada (Kovacevic et al., 2016). For twenty seven of the isolates from the present study the whole genome sequences were available, and the presence of *emrE* was investigated. The gene *emrE* was not present in any of these Norwegian strains.

We are not aware of other studies that report residues of QAC on equipment after sanitation. In Norway it is common to rinse surfaces (equipment, machines, floor, walls etc.) with water of drinking water quality after disinfection. The presence of BC on surfaces after sanitation demonstrated that the rinsing steps were not sufficient to remove all QAC residues from the surfaces. Residual disinfectants may be transferred from equipment/machines to food during processing and there has been an increased focus on QAC residues in food as the EU has applied regulations for maximum residue levels in foods for the QACs benzalkonium chloride and didecyldimethylammonium chloride (0.01–0.1 mg/kg, (European Commission, 2005, 2014)). Residues of QAC have been found on various types of food, including meat products, dairy products, fruits and nuts (European Food Safety Authority, 2013; Bertuzzi and Pietri, 2014). While residues on some types of food like fruits may be a result of the use of QACs in plant protection products, or for milk products the use of QAC for washing cow udders, residues in meat products strengthens the theory that residual QAC on food contact surfaces after sanitation are not uncommon.

It must be noted that, in addition to insufficient rinsing after disinfection, the use of inadequate QAC concentrations or applying the disinfectant on wet surfaces, hence diluting the disinfectant, may also lead to niches with sub-lethal QAC concentrations, where *L. monocytogenes* harbouring *qacH*/*bcrABC* may have a growth advantage.

Results from growth and survival studies indicated that harbouring *qacH* or *bcrABC* may contribute to increased fitness in situations where *L. monocytogenes* are exposed to BC-concentrations at the border of growth inhibition but not at lethal concentrations. The presence of *qacH*/*bcrABC* did not lead to higher survival after exposure to BC, neither in biofilm or in suspensions. As also shown before, *L. monocytogenes* in biofilms was more tolerant to QAC than in suspension (Romanova et al., 2007). Several different mechanisms not associated with *qacH*/*bcrABC* may explain the increase tolerance of biofilms cells to antimicrobial compounds, such as limited diffusion of biocides in biofilms, neutralization of biocides by the biofilm matrix, the presence of tolerant dormant cells and different gene expression in biofilms (Costerton et al., 1995; Van Acker et al., 2014).

When the commercial disinfectant was diluted to 1 ppm BC in the laboratory, *L. monocytogenes* strains without *qacH* or *bcrABC* had an increased lag phase compared to strains containing either of these genes; however water samples from processing plants containing BC with C-12 chain length with concentration as high as 6.7 ppm did not affect growth of the same isolates. It cannot be excluded that the water samples in addition to BC residues contained substances that may lower the inhibitory effect of BC, such as soil (proteins, lipids) or divalent cations etc. (Chinard, 1948). Also it may be difficult to compare the antibacterial effect of BC from different producers, as the ratio between alkyl groups with different chain length may vary, and it is known that the antibacterial effect of BC depends on the chain lengths of these groups (Gilbert and Moore, 2005).

It has been shown by others that sensitive strains of *L. monocytogenes* may adapt to QAC including BC during growth at sub-inhibitory concentrations (Aase et al., 2000; Kastbjerg and Gram, 2012; Lunden et al., 2003; Romanova et al., 2006; To et al., 2002). It has also been shown that strains adapted to BC overexpressed the chromosomally encoded multidrug efflux pump MdrL (Mereghetti et al., 2000; Romanova et al., 2006). The presence of *mdrL* was confirmed in the 27 strains from the present study, where the genome sequences were available. The increased tolerance of *L. monocytogenes* due to adaptation was reported to be stable for up to 7 or 28 days without exposure to the disinfectants (Aase et al., 2000; Lunden et al., 2003). Our results suggest that such adaptation is rarely occurring in the food processing environments as all wild type isolates of *L. monocytogenes* without qacH/bcrABC were sensitive to BC. As the exposure time required for adaptation to BC in laboratory experiments (20 − 100h) exceeded time spans where water remains and bacteria may grow in the food industry e.g. between cleaning and start of production, the practical significance of laboratory adaptation may be low.

It has frequently been shown that *L. monocytogenes* is able to appear and survive in production environments; it is, however, yet an open question why some strains are frequently isolated over prolonged periods of time, while others merely appear sporadically. A statistical method described by Malley et al. (2013) was used to characterize the various MLVA profiles as persistent or sporadic depending on isolation frequency. Of the 26 analyzed MLVA profiles, 10 were characterized as persistent according to this method. Four of these contained *qacH* or *bcrABC*, while 3 out of 16 sporadic MLVA profiles contained *qacH*/*bcrABC*. There were no statistical correlation between the prevalence of QAC tolerance genes and persistence. There are other studies that have reported increased QAC tolerance among persistent *L. monocytogenes* (Aase et al., 2000; Fox et al., 2011; Ortiz et al., 2014); however *L. monocytogenes* can also be established in processing plants where other disinfectants than QAC are employed. Even if tolerance to QAC may be a factor for increased growth and subsequently persistence in the production environment, there must also be other factors important for such persistence. It has been suggested that persistent strains have increased capacity for adherence or biofilm formation compared to sporadic strains, but other findings do not support this hypothesis (Ferreira et al., 2014).

It must be noted that cleaning and disinfection regimes (including the type of disinfectant used) in several facilities were adjusted or

changed during the time of the samplings and the impact of these changes on persistence could not be determined. In general, alternating with another type of disinfectant, e.g. an oxidative type like peracetic acid or chlorine once a week may decrease the risk of selection of *L. monocytogenes* tolerant to QAC.

Although the selection of strains for which whole genome sequences are publicly available is clearly biased towards certain STs, we did observe that the prevalence of *qacH* genes was higher in the set of representative isolates from meat- and salmon industry examined in the current study compared with in the public genome sequences (22% vs. 5%). As many of the public available sequences represent human clinical strains, this may indicate a higher *qacH* prevalence among food associated strains. This is supported by a study by (Müller et al., 2013), where 10 out of 59 isolated from food or food processing environments were *qacH* positive, while none out of 22 human isolates contained *qacH*. In contrast, the prevalence as well as the extent of phylogenetic distribution for strains carrying *bcrABC* was higher among the public genomes than in the set of isolates from this study (prevalence 43% vs. 8%, respectively, with only one ST from the current study out of eight harbouring *bcrABC*) (Fig. 1). Müller et al. (2013) found 6.7% *bcrABC* positives among food associated isolates. The scattered distribution of isolates containing *qacH* or *bcrABC* genes on different phylogenetic lineages of *L. monocytogenes* is not unexpected, since these genes are known to reside on transposons which are mobile genetic elements (Elhanafi et al., 2010; Müller et al., 2013). With one exception, isolates from the current study with the same MLVA profile had the same distribution of *qacH* and *bcrABC*. Two potential explanations may account for this observation: Firstly, the conservation of the mobile genetic elements onto which the BC resistance genes reside may confer an advantage to strains in the food industry environment, resulting in selection of strains retaining these genes. Alternatively, the similar profiles for BC resistance genes within each MLVA profile are a reflection of a very close evolutionary relationship between strains.

The difference in susceptibility to BC between isolates harbouring *qacH* corresponded to SNPs in the *qacH* gene, out of which one SNP resulted in a cysteine/serine difference at amino acid 42 of the putative QacH. The same SNPs and the same correlation to BC tolerance in a MIC assay was previously reported by Müller et al. (2013). They, however, argued that as the SNPs did not cause substitutions in residues known to be essential for the function of related proteins, they were not the cause of the observed differences in BC tolerance between isolates. Further work involving cloning and expression of the different variants of *qacH* would be needed to resolve this issue.

A total of 680 *L. monocytogenes* isolates were collected from the Norwegian meat and salmon industry. Further characterization by MLVA and MLST showed that the isolates could be grouped in several profile groups however, MLVA showed a higher resolution compared to MLST. It is however expected that in the near future whole genome sequence typing will replace conventional molecular subtyping methods also in surveillance studies like the one performed in the current investigation (Gardy et al., 2015; Kwong et al., 2016; Nyarko and Donnelly, 2015).

The present study supports earlier reports that genes leading to increased tolerance to QAC are commonly found among *L. monocytogenes* isolates from food processing environments. These efflux genes can make *L. monocytogenes* more fit to grow in the presence of residual concentrations of QAC in niches where the bacterium is exposed to sublethal concentrations of disinfectants. Thorough rinsing after disinfection or alternating with another type of disinfectant may avoid an accumulation of QAC-tolerant isolates in the food industry.

Acknowledgments

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