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

Trond Møretrø, Bjørn C.T. Schirmer, Even Heir, Annette Fagerlund, Pernille Hjemli, Solveig Langsrud

**A R T I C L E   I N F O**

Article history:
Received 18 May 2016
Accepted 19 October 2016
Available online 21 October 2016

**Keywords:**
Listeria monocytogenes
Quaternary ammonium compound
Tolerance

**A B S T R A C T**

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.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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 (Göttlieb 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 production 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 typically 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). QAC, BcR, and BcC 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, Tn56188, 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 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://biggsdb.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 (Elhanna 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 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 × 10⁶ 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 × 10⁶ 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. Bacterial 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 dH₂O (control) or BC (10 ppm final concentration) resulting in a final cell concentration of 10⁷ 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. Bacterial 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 dH₂O 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 dH₂O. Coupons were then transferred to glass tubes containing 6 ml of dH₂O (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 (Branson 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 QA resistance genes bcrABC or qacH at BC concentrations and conditions that may be present after sanitation. The five strains tested included one bcrABC positive (MF4624orf42), one isolate with qacH with cystine in position 42 (MF5380orf42cys), one isolate with qacH with serine in position 42 (MF5376orf42s). 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).
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). 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.
The prevalence of \( qacH \) in the 352 publicly available sequences was 5\% (compared with 22\% in our isolates), while 43\% of sequenced strains contained \( \text{bcrABC} \) (compared with 8\% for our isolates). With isolates from 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 genetic determinants for BC tolerance were determined, were subjected to MIC experiments to determine sensitivity to BC. The MICs for the 70 isolates containing neither \( qacH \) nor \( \text{bcrABC} \) were ≤ 5 ppm BC while isolates containing \( qacH \) (23 isolates) and \( \text{bcrABC} \) (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 and 6 (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 encoded by the central 109 amino acids (aa) of \( QacH \) for these isolates. The alignment presented in Fig. 2 also includes the \( QacH \) sequences encoded by the central 109 amino acids (aa) of \( QacH \) for these isolates.

### Table 3

<table>
<thead>
<tr>
<th>Concentration of Benzalkonium chloride (BC)</th>
<th>Effect on ( L. ) monocytogenes</th>
<th>Sample no.</th>
<th>BC-12</th>
<th>BC-14</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ppm</td>
<td></td>
<td>M1-9</td>
<td>2.5b</td>
<td>0.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 ppm</td>
<td></td>
<td>M1-11</td>
<td>2.6</td>
<td>0.90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.2 ppm</td>
<td></td>
<td>M1-12</td>
<td>6.7</td>
<td>0.85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1 ppm</td>
<td></td>
<td>M2-1</td>
<td>0.23</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.05 ppm</td>
<td></td>
<td>M2-2</td>
<td>0.13</td>
<td>0.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.025 ppm</td>
<td></td>
<td>M2-9</td>
<td>341</td>
<td>124</td>
<td>+d</td>
<td>-</td>
</tr>
<tr>
<td>0.01 ppm</td>
<td></td>
<td>M2-11</td>
<td>14</td>
<td>2.7</td>
<td>+e</td>
<td>-</td>
</tr>
<tr>
<td>0.005 ppm</td>
<td></td>
<td>M2-14</td>
<td>196</td>
<td>51</td>
<td>+e</td>
<td>-</td>
</tr>
</tbody>
</table>

\[d\] Indicates results 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 (\( \text{bcrABC} \)), MF5380- \( \text{qacH} \), MF5634 (\( \text{bcrABC} \)), MF5376 (\( \text{bcrABC} \)), and MF4993 (\( \text{bcrABC} \)). Ctrl = control isolate without \( qacH \) or \( \text{bcrABC} \).

\[e\] Increased lag phase of growth of \( L. \) monocytogenes MF5376 (\( \text{bcrABC} \)) and MF4993 (\( \text{bcrABC} \)).

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: ST2, MLVA profiles 5 and 6: \( QacH \) from the three MLVA profile 6 (ST21) isolates MF3858, MF5216, and MF5634, and from one MLVA profile 5 (ST121) isolate (MF3908). In addition, the following publicly available ST21 L. monocytogenes WGS encode this \( QacH \) variant: 6179 (HG813249), Lm_1880 (K074110), 3251 (JY00100006), N531 (AAXD01000004), 4423 (CRBR010000008), MOD1_L589 (JRAZ01000002), S2_3 (JWH01000031), S2_2 (JWH01000009), S10_1 (JWH0100009), and S10_3 (JWH01000037). ST7 and ST2, MLVA profile 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); JY00100008, and Lm_1824 (ST2; K074021), ST21, MLVA profile 7: \( QacH \) from the MLVA profile 7 (ST21) isolate (MF5095). HPB2262-ST2: \( QacH \) from the publicly available WGS from ST2 L. monocytogenes strain HPB2262 (AA1D000003333), ST5: \( QacH \) from the publicly available ST5 L. monocytogenes WGSs from FSL R8-5124 (JWHR01000002), FSL R8-6480 (JWHR01000002), FSL R8-7153 (JPBD01000002), and FSL R8-7478 (JPR010000001). 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 highest concentrations of BC were found in the three water samples with adverse effects against L. monocytogenes (Table 3). In the third sample (M2-11), the lag phase of isolates not 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 period. 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, growth after ca 10 h lag phase was observed at 0.001% DesQA for previously adapted isolates cultured without QAC for 10–60 generations, 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).
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/bcrABC 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_ is 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 _L. monocytogenes_ can grow are commonly 20–300 ppm. The question has hence been raised whether this tolerance level has any practical relevance in the food industry, namely when QAC containing disinfectants are not rinsed off properly after sanitation.

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 on 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 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. Thorring after disinfection or alternating with another type of disinfectant may avoid an accumulation of QAC-tolerant isolates in the food industry.

Acknowledgments

The authors wish to thank Anette Wold Åsli and Tove Maugesten at Nofima for excellent technical assistance. Lin Thorstensen Brandal and Bjørn Arne Lindstedt at the Norwegian Institute of Public Health are acknowledged for MLVA profiling. We thank the team of curators of the Institut Pasteur MLST system (Paris, France) for importing novel alleles, profiles and/or isolates at http://www.pasteur.fr/mlst. This work was founded by the Norwegian Research Funding for Agriculture and Food Industry (grant no. 207765) and The Norwegian Seafood Research Fund (grant no. FHF-900521).

References


European Food Safety Authority, 2013. Evaluation of monitoring data on residues of didecyl(dimethyl)ammonium chloride (DDAC) and benzalkonium chloride (BAC). EFSA Supporting Publication 2013, p. 30 (EN-483).


European Reference Laboratories for Residues of Pesticides, 2014, Analysis of BACs and DDAC in Milk Using QuChERS Method and LC-MS/MS. EURIL SRM. Version 2 (05.05.2014).


Gaulin, C., Ramsay, D., Bekal, S., 2012. Widespread listeriosis outbreak attributable to pasteurized cheese, which led to extensive cross-contamination affecting cheese retailers, Quebec, Canada. 2008. J. Food Prot. 75, 71–78.
