RESEARCH LETTER

The Listeria monocytogenes transposon Tn6188 provides increased tolerance to various quaternary ammonium compounds and ethidium bromide

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Abstract
Tolerance of the foodborne pathogen Listeria monocytogenes to sublethal concentrations of disinfectants has been frequently reported. Particularly, quaternary ammonium compounds (QACs) such as benzalkonium chloride (BC) are often used in disinfectants and also as antiseptics in food industry and hospitals. Recently, we described Tn6188, a novel transposon in L. monocytogenes harbouring the transporter QacH, a molecular mechanism leading to increased tolerance to BC. In this study, we investigated the presence of Tn6188 within the genus Listeria spp. Our screening indicates that the distribution of Tn6188 may be limited to L. monocytogenes. We confirm that QacH is responsible for the observed increase in tolerance by complementation of a qacH deletion mutant and introducing qacH in a Tn6188 negative strain. We investigated the transporter’s substrate spectrum by determining minimal inhibitory concentrations (MICs) and showed that QacH also confers higher tolerance towards other QACs and ethidium bromide (EtBr). This result was supported by increased expression of qacH in the presence of the various substrates as determined by quantitative reverse transcriptase PCR (qRT-PCR). In addition, we detected expression of a Tn6188 transposase gene and circular forms of Tn6188, suggesting activity and possible transfer of this transposon.

Introduction
Recently, we described the novel transposon Tn6188 in Listeria monocytogenes strains 4423 and 6179 (Mueller et al., 2013). The three Tn6188 transposase genes tnpABC were highly similar to transposases of Tn554-like transposons. Tn6188 encodes QacH, a transporter belonging to the small multidrug resistance protein family (SMR) highly similar to Smr/EmrE/Qac proteins which have been shown to increase tolerance against different disinfectants in, for example, Staphylococcus aureus (Smr/Qac) and Escherichia coli (EmrE) (Bay et al., 2008). Screening of 91 different L. monocytogenes strains revealed presence of Tn6188 in 11% of the strains. Strains harbouring Tn6188 had significantly higher benzalkonium chloride (BC) minimal inhibitory concentrations (MICs) than qacH deletion mutants (Mueller et al., 2013).

Quaternary ammonium compounds (QACs) such as BC, benzethonium chloride (BZT), cetramethylammonium bromide (CTAB), domiphen bromide, cetylpyridinium chloride monohydrate (CPC) and dodecyltrimethylammonium bromide (DTAB) are frequently used as antiseptics and in disinfectants in hospitals, the food industry and veterinary medicine (McDonnell & Russell, 1999; Bjorland et al., 2001; Marriott & Gravani, 2006; Fox et al., 2011). QACs are membrane-active agents leading to membrane disorganization and disruption of the bacterial cell walls causing cytosolic leakage and enzyme inhibition (McDonnell & Russell, 1999; Marriott & Gravani, 2006; Fox et al., 2011).

Tolerance of L. monocytogenes to sublethal concentrations of QACs has frequently been reported (Aase et al., 2000; Mereghetti et al., 2000; Romanova et al., 2002, 2006; To et al., 2002; Soumet et al., 2005; Mullapudi et al., 2008; Elhanafi et al., 2010; Fox et al., 2011; Rakic-Martinez, 2011).
et al., 2011), but so far, besides Tn6188, only the bcrABC resistance cassette, providing increased tolerance to BC due to the SMR transporter BcrBC, has been described (Elhanafi et al., 2010; Dutta et al., 2013). The efflux pumps MdrL and Lde, which belong to the major facilitator superfamily (MFS), also seem to be involved in tolerance of L. monocytogenes to BC, ethidium bromide (EtBr) and other substances such as antibiotics (Mata et al., 2000; Romanova et al., 2002, 2006; Godreuil et al., 2003; Soumet et al., 2005; Rakic-Martinez et al., 2011). Furthermore, genomic data revealed the presence of many putative transporters in L. monocytogenes EGDe (Glaser et al., 2001), some of which might contribute to resistance phenotypes. Kuenne et al. (2010) found several genes involved in multidrug efflux, for example SMR-like genes, on different Listeria plasmids. It is essential to gain more knowledge on possible resistance mechanisms as L. monocytogenes is an opportunistic foodborne pathogen which can cause severe listeriosis with a high mortality rate particularly in immunocompromised patients, pregnant women, elderly and infants (Allerberger & Wagner, 2010).

The aim of this study was to perform a more detailed analysis of Tn6188 and QacH by: (1) investigating the distribution of Tn6188 among other Listeria spp. than L. monocytogenes; (2) complementation of a qacH deletion mutant; (3) analysing the transporter’s putative substrate spectrum by MIC determinations and qRT-PCR; and (4) investigating the activity and mobility of Tn6188 using qRT-PCR targeting tnpA.

Materials and methods

PCR screening for Tn6188 in Listeria spp.

We screened all non-L. monocytogenes isolates of the Listeria strain collection of our institute for the presence of Tn6188. Strains have been isolated from different sources and in different years. In total, 114 Listeria spp., including 55 L. innocua, 42 L. seeligeri, 7 L. welshimeri and 10 L. ivanovii strains (Supporting Information, Table S1) were used; the strains were selected independent of BC-tolerant phenotypes and grown overnight in BHI at 37 °C with 125 r.p.m. shaking. DNA was isolated from 2 mL culture using the DNeasy Blood and Tissue Kit (Qiagen) according to the instructions of the manufacturer. PCR primers targeting the radC gene of the species L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri and L. ivanovii, into which Tn6188 is integrated in L. monocytogenes, were designed (radC_sp_fwd: 5’-TARC CTTTTTCTTTTACCTT-3’, radC_sp_rev: 5’-CAYCCDAG AGARGTDTTATGTTT-3’) and yield a PCR amplicon of 5310 bp in case Tn6188 is present and of 200 bp when Tn6188 is absent. PCR conditions were as follows: 1 pmol μL⁻¹ of each primer, 2 mM MgCl₂, 1 mM dNTP-Mix, 0.625 U Platinum Taq DNA polymerase (Life Technologies). PCR cycling conditions were as follows: 5 min at 95 °C; 30 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 165 s; 72 °C for 5 min. Additionally, strains were screened with the primers qacH_fwd and qacH_rev targeting the qacH gene on Tn6188 and yielding a PCR amplicon of 366 bp (Mueller et al., 2013). Negative and positive controls were included in all PCR reactions. The presence and size of amplification products were checked with agarose gel electrophoresis and EtBr staining.

Complementation of the L. monocytogenes 4423 qacH deletion mutant

QacH of L. monocytogenes 4423 was amplified with the primers QacH_CompF (5’-CGGCCATGGCATATCTATA TITAGCA-3’) and QacH_CompR (5’-CCGTCTAGAGAC TCATAGCATATATAAAATAA-3’), which contain restriction sites for NcoI and XbaI and yield a 439 bp amplicon. For PCR, the following concentrations were used: 0.2 pmol μL⁻¹ of each primer, 1 mM dNTP-Mix; PfuUltra II Fusion HS DNA Polymerase and buffer (Agilent Technologies) were used according to the manufacturer’s protocol. PCR cycling conditions were as follows: 5 min at 95 °C; 40 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 20 s; 72 °C for 5 min. The plasmid pNZ44 and the purified PCR product were digested with XbaI/NcoI (ThermoScientific) (McGrath et al., 2001), purified and equal amounts were ligated with T4-Ligase (ThermoScientific). The ligation product (pNZ44-qacH) was transformed into competent E. coli cells (StrataClone SoloPack, Agilent Technologies) and transformants were selected on BHI agar containing 10 μg mL⁻¹ chloramphenicol (BHI-Cm; Sigma-Aldrich). pNZ44-qacH was then transformed into the competent L. monocytogenes 4423 qacH deletion mutant (an isogenic mutant generated in our previous study) and R479a using the following parameters: voltage 2 kV, resistance 400 Ω, capacity 25 μF (Mueller et al., 2013). After electroporation, 1 mL of BHI with 0.5 M sucrose was added and incubated for 1 h at 30 °C. Cells were then selected on BHI-Cm, and presence of pNZ44-qacH was confirmed by PCR.

Determination of minimum inhibitory concentrations (MICs)

For MIC determination, L. monocytogenes wildtype strain 4423, the qacH deletion mutant, and the complemented qacH deletion mutant strain were used. MIC determinations were performed as previously described (Soumet et al., 2005; Elhanafi et al., 2010; Mueller et al., 2013).
The following substrate concentrations were used: BC (Sigma-Aldrich) and BZT (Sigma-Aldrich): 0–30 mg L\(^{-1}\) (intervals: 5 mg L\(^{-1}\)); CTAB (BioChemika), domiphen bromide (Sigma-Aldrich) and CPC (BioChemika): 0–40 mg L\(^{-1}\) (intervals: 5 mg L\(^{-1}\)); DTAB (BioChemika): 0–120 mg L\(^{-1}\) (intervals: 5–10 mg L\(^{-1}\)); EtBr (Sigma-Aldrich): 0, 20, 25, 30, 40, 50, 85 and 100 mg L\(^{-1}\); Weiquat (TC Tensid Chemie): 0%, 0.001%, 0.005%, 0.01%, 0.013%, 0.02% and 0.027% (v/v) (corresponding to 1.5, 7.5, 15, 20, 30 and 40 mg L\(^{-1}\) BC); BC MICs were also determined for L. monocytogenes R479a and L. monocytogenes R479a containing pNZ44-qacH. The MIC was defined as the lowest assessed concentration of the various substrates which prevented growth. Experiments were performed in three or six biological independent replicates. Mean values and standard deviations (SD) of all performed replicates (either three or six replicates) were calculated (Microsoft Excel\textsuperscript{®} 2007).

**Expression of the qacH and tnpA genes**

**RNA isolation, transcription into cDNA**

To investigate the expression of qacH and tnpA in the presence of different QACs, one colony of L. monocytogenes 4423 was grown overnight in 24 mL BHI at 37 °C with shaking (125 r.p.m.). The bacterial culture was adjusted to an OD\(_{600}\) of 0.2 in a final volume of 200 mL BHI broth. Cells were grown at 20 °C with shaking (125 r.p.m.) to an OD\(_{600}\) of c. 0.7. Cultures were split in 12 parts of 15 mL each and incubated for 30 min at 20 °C with shaking with the following concentrations of the tested substrates: 5 mg L\(^{-1}\) BZT; 3 mg L\(^{-1}\) CTAB; 5 mg L\(^{-1}\) domiphen bromide; 2 mg L\(^{-1}\) CPC; 100 mg L\(^{-1}\) DTAB; 0.005% Weiquat; 50 mg L\(^{-1}\) EtBr and without any substrate. Cells were centrifuged (3220 g, 20 °C, 10 min) and washed with 1 mL RNAlater (Life Technologies). Pellets were resuspended in 1 mL RNAlater and stored at 4 °C until RNA isolation. RNA isolation was performed using TRIzol Reagent (Life Technologies) according to a protocol described in (Mueller et al., 2013). DNA was digested and absence of DNA was confirmed by PCR (Mueller et al., 2013). The RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) was used for cDNA synthesis as previously described (Mueller et al., 2013). Experiments were performed in three or five independent biological replicates.

**Quantitative reverse transcriptase (qRT)-PCR**

We used the 16S rRNA gene as an internal reference (Tasara & Stephan, 2007). 0.4, 4 and 20 ng cDNA were used as template in each reaction for 16S rRNA- gene, qacH- and tnpA qRT-PCR, respectively. Primers, cycling conditions and PCR concentrations for the qacH and 16S rRNA gene qRT-PCR were the same as previously described (Mueller et al., 2013). For tnpA, qRT-PCR primers were designed: tnpA_fwd (5′-GAT-ACGAGGGATAATACCTCTCTCATG-3′) and tnpA_rev (5′-CAAAAATTGGTGGATTGTTAAAGAATC-3′) yielding a PCR product of 401 bp. Concentrations and cycling conditions were the same as for the 16S rRNA gene qRT-PCR except for an annealing temperature of 60 °C. A dilution series of genomic DNA from L. monocytogenes 4423 (1–10\(^{-4}\) ng \(\mu\)L\(^{-1}\) for 16S rRNA- gene and qacH qRT-PCR and 1–10\(^{-5}\) for tnpA qRT-PCR) was used as an internal amplification control and for calculation of primer efficiencies (1.7–1.96 for qacH, 1.58–1.62 for 16S rRNA gene and 1.68 for tnpA). Data were analysed using Mx300P MXPRO software (Stratagene). Each sample was measured in duplicate (technical replicates), and mean values of these duplicates were used for relative quantification using the comparative Ct method (calculated with Microsoft Excel\textsuperscript{®} 2007 software). All x-fold changes of the replicates of one substance were compared to the x-fold changes of the control with the nonparametric U-test (Mann–Whitney U-test) for two independent random samples using the software STATISTICA. This was carried out for all substances used; substances were not compared with each other. '2 \(\times\) 1 exact P'-values < 0.05 were considered to be significant.

**Detection of circular forms of Tn6188**

Listeria monocytogenes 4423 was grown overnight in BHI at 37 °C with 125 r.p.m. shaking. DNA was isolated from 2 mL culture using the DNeasy Blood Tissue Kit (Qiagen) according to the instructions of the manufacturer. A forward primer Tn_circ_fwd (5′-ACTTTCATCTTTCCA CCACC-3′) and a reverse primer Tn_circ_rev (3′-ATCT TCAACTAACGGGAGCG-5′) were designed, which result in a 367 bp amplicon if circular forms are present (Fig. S1). Concentrations and cycling conditions used were the same as for the radC-PCR except that 0.2 pmol \(\mu\)L\(^{-1}\) of each primer was used, an annealing temperature of 54 °C and elongation for 20 s. The PCR product was purified and sequenced (LGC Genomics).

**Results and discussion**

In our previous study, Tn6188 was found in 11% of 91 screened L. monocytogenes strains from various sources (Mueller et al., 2013). Here, we screened 114 Listeria spp. including: 55 L. innocua, 42 L. seeligeri, 7 L. welshimeri and 10 L. ivanovii strains of different sources by PCR but could not detect any strain harbouring Tn6188 (Table S1).
This strongly suggests that Tn6188 is primarily present in *L. monocytogenes*, but more strains of the different *Listeria* spp. should be screened to strengthen this hypothesis. Particularly *L. innocua*, but also *L. seeligeri*, *L. welshimeri* and *L. grayi* have been isolated from food and food processing environment (Williams et al., 2011; Rahimi et al., 2012; Hellberg et al., 2013), which might allow transfer of Tn6188 between different *Listeria* species.

We confirm that QacH is responsible for the increased tolerance to BC observed for *L. monocytogenes* 4423 and other *L. monocytogenes* strains harbouring Tn6188. We complemented the *L. monocytogenes* 4423 qacH deletion mutant by introducing pNZ44-qacH, leading to constitutive expression of *qacH*. The complemented strain showed higher BC MICs (20 ± 0 mg L⁻¹) than the deletion mutant (10 ± 0 mg L⁻¹), although lower than BC MICs of the wildtype strain (30 ± 0 mg L⁻¹) (Fig. 1). Lower MICs of the complemented strain compared to the wildtype might, for example, be caused by different promoters. Furthermore, we introduced pNZ44-qacH to the Tn6188-lacking strain *L. monocytogenes* R479a and observed a similar effect: the BC MIC of *L. monocytogenes* R479a + pNZ44-qacH was 20 ± 0 mg L⁻¹ and of the wildtype strain R479a 11.7 ± 2.9 mg L⁻¹ (Fig. 1).

To get more insight into the putative substrate spectrum of QacH, we determined MICs for seven substances which have been reported to be transported by other Qac/Smr/EmrE proteins (Bay et al., 2008). Besides the dye EtBr – a therapeutic used in veterinary medicine against trypanosomiasis in cattle – all analysed substances are QACs (Radostits et al., 2007; Roy Chowdhury et al., 2010). Additionally, Weiquat, a disinfectant containing BC and used for ‘cleaning-in-place’ (CIP), an automated cleaning system for equipment in food production processes, was analysed (Marriott & Gravani, 2006). Although the tested QACs exhibit structural differences (Fig. S2), tolerance towards all QACs was increased by the presence of QacH: Higher MICs were determined for all QACs and Weiquat as well as for EtBr using *L. monocytogenes* wildtype strain 4423 and the complemented deletion mutant compared to ΔqacH 4423. However, *L. monocytogenes* 4423 ΔqacH + pNZ44-qacH showed slightly lower MICs than the wildtype strain (Fig. 2). Differences in the MICs of the wildtype compared to the mutant vary between 1.6 and 2.8-fold (Fig. 2). The Weiquat MIC of the wildtype strain was 0.013 ± 0% (containing 20 mg L⁻¹ BC) and of the ΔqacH strain 0.008 ± 0.003% (containing 12 mg L⁻¹ BC). Results are thus similar to those observed for BC alone. A working solution of Weiquat contains c. 150 mg L⁻¹ BC. Consequently, appropriate Weiquat concentrations will also kill *L. monocytogenes* harbouring Tn6188. The same is true for other QACs tested: for example, the maximum recommended working concentration for QACs in food processing plants is 200 mg L⁻¹ which is significantly higher than the BC MIC observed for *L. monocytogenes* 4423 (30 ± 0 mg L⁻¹) as well as the DTAB MIC, which was the highest MIC observed in our study (112.5 ± 5 mg L⁻¹) (Ferreira et al., 2014). Nevertheless, when disinfectant concentrations in the food processing environment are reduced, for example, due to biofilms, debris, dosage failure or in hard to-reach-areas (Best et al., 1990; Saa I busquiza et al., 2011; Ferreira et al., 2014), harbouring Tn6188 might be advantageous to survive sublethal disinfectant concentrations.

Our study is in accordance with other studies reporting a higher tolerance to various QACs and EtBr due to Qac/Smr/EmrE-like proteins with MICs in a similar range (Pau lsen et al., 1993, 1995; Yerushalmi et al., 1995; Bjorland et al., 2001; Nishino & Yamaguchi, 2001; Fuentes et al., 2005; Bay et al., 2008). A recent phenotypic characterization of *L. monocytogenes* 6179 also showed increased tolerance to BZT, CPC, DTAB and domiphen bromide (Fox et al., 2011). Furthermore, other QacH-like SMR proteins can be found in *L. monocytogenes* genomes, for instance two proteins highly similar to the *Bacillus subtilis* SMR transporters YkkC and YkkD. Presence of the gene pair ykkCD was shown to increase tolerance to, for example, EtBr, CPC, crystal violet and tetracycline in *E. coli* (Jack et al., 2000). Increased expression of qacH, ykkC, bmrB, ykkD and other transporters as well as many genes involved in various biological functions has recently been reported when *L. monocytogenes* 6179 was exposed to sublethal concentrations of BZT (Casey et al., 2014).

qRT-PCR showed significantly higher expression of qacH in *L. monocytogenes* 4423 after 30 min growth in

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**Fig. 1.** BC MICs for *Listeria monocytogenes* wildtype strain 4423 (4423 WT), the qacH deletion mutant (4423 ΔqacH) and the complemented deletion mutant (4423 ΔqacH + pNZ44-qacH), *L. monocytogenes* wildtype strain R479a and the transformed *L. monocytogenes* R479a + pNZ44-qacH (R479a + pNZ44-qacH). Mean values and standard deviations of the three independent biological replicates are presented. Bars without error bars are due to the same numerical results in all replicates.
the presence of BZT, CTAB, domiphen bromide, DTAB, Weiquat and EtBr and thus supported increased tolerance due to QacH (Fig. 3). The increase in expression of qacH in the presence of CPC was not statistically significant. An additional and more thorough analysis including whole transcriptome data could provide more reliable results and insights into the mechanism of QAC tolerance. Our data may be limited using only the 16S rRNA gene as housekeeping gene. Using the 16S rRNA gene as an internal reference for qRT-PCR is controversially discussed. Usage of more than one reference gene will improve reliability; nevertheless, the 16S rRNA gene was the most stably expressed housekeeping gene in *L. monocytogenes* in a study conducted by Tasara and Stephan (Tasara & Stephan, 2007). Furthermore, our results are confirmed by a recent study using RNAseq of *L. monocytogenes* 6179 (harbouring a Tn6188 copy, which is identical to Tn6188 from strain 4423), which showed a fourfold upregulation of qacH in the presence of BZT (Casey et al., 2014) – similar to what we observe here. Although qRT-PCR supports our MIC data that QacH’s substrate spectrum comprises various QACs, indirect effects during deletion and complementation may influence MICs. Additional experiments such as heterologous expression in *E. coli* or proteoliposomes could corroborate our results.

Expression of tnpA indicates possible transpositional activity and hence mobility and potential transfer of Tn6188, which might lead to a distribution of QacH. qRT-PCR showed that tnpA was expressed under all conditions tested (Fig. 4), also in our control sample (without substrate), which is also true for qacH. Although a numerical increase in expression can be seen in the presence of the various substances, in particular for domiphen bromide and EtBr, none of the changes is statistically significant. Insertion of a transposon can increase its host’s fitness in the long run by introducing variation (Nagy & Chandler, 2004) or, for example, by carrying resistance genes, but it can also be detrimental as transposons can disrupt functionally important genes (Nagy & Chandler, 2004). In addition to the detection of tnpA-transcription, we could also detect circular forms of Tn6188 which is...
further supporting the hypothesis that Tn6188 is active (data not shown). Circular forms have been detected for the related transposons Tn558, Tn559 and Tn5406 (Harrow et al., 2002; Kehrenberg & Schwarz, 2005; Kadlec & Schwarz, 2010).

In conclusion, we could prove that QacH increases tolerance to BC by complementing the L. monocytogenes 4423 deletion mutant and inserting qacH in a Tn6188 negative strain. Furthermore, QacH also confers increased tolerance against five other QACs (BZT, CTAB, domiphen bromide, CPC, DTAB), EtBr and the sanitizer Weiquat, resulting in higher MIC values as well as in increased expression of the transporter. QACs are frequently used sanitizers in food production industry (Marriott & Gravani, 2006). Thus, Tn6188 could be an advantage for persistence in food production environments, for example, when sanitizer concentrations are too low (e.g. reduced by food debris, biofilms or wrong dosage) (Best et al., 1990; Saa Ibusquiza et al., 2011). Expression of tnpA and circular forms of Tn6188 suggest transpositional activity and possible dispersal. Transfer experiments would be needed to confirm this hypothesis. However, in our screening of 114 Listeria spp. strains, Tn6188 was not detected, suggesting that it might be restricted to L. monocytogenes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Primer binding sites for the detection of circular forms and the resulting PCR product.

Fig. S2. Structures of the tested substrates.

Table S1. Sources of Listeria spp. used for PCR screening.