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The influence of subminimal inhibitory concentrations of benzalkonium chloride on biofilm formation by *Listeria monocytogenes*

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ABSTRACT

Disinfectants, such as benzalkonium chloride (BAC), are commonly used to control *Listeria monocytogenes* and other pathogens in food processing plants. Prior studies have demonstrated that the resistance to BAC of *L. monocytogenes* was associated with the prolonged survival of three strains of molecular serotype 1/2a in an Iberian pork processing plant. Because survival in such environments is related to biofilm formation, we hypothesised that the influence of BAC on the biofilm formation potential of *L. monocytogenes* might differ between BAC-resistant strains (BAC-R, MIC ≥ 10 mg/L) and BAC-sensitive strains (BAC-S, MIC ≤ 2.5 mg/L). To evaluate this possibility, three BAC-R strains and eight BAC-S strains, which represented all of the molecular serotype 1/2a strains detected in the sampled plant, were compared. Biofilm production was measured using the crystal violet staining method in 96-well microtitre plates. The BAC-R strains produced significantly ($p < 0.05$) less biofilm than the BAC-S in the absence of BAC, independent of the rate of planktonic growth. In contrast, when the biofilm values were measured in the presence of BAC, one BAC-R strain (S10-1) was able to form biofilm at 5 mg/L of BAC, which prevented biofilm formation among the rest of the strains. A genetic determinant of BAC resistance recently described in *L. monocytogenes* (Tn*6188*) was detected in S10-1. When a BAC-S strain and its spontaneous mutant BAC-R derivative were compared, resistance to BAC led to biofilm formation at 5 mg/L of BAC and to a significant ($p < 0.05$) stimulation of biofilm formation at 1.25 mg/L of BAC, which significantly ($p < 0.05$) reduced the biofilm level in the parent BAC-S strain. Our results suggest that the effect of subminimal inhibitory concentrations of BAC on biofilm production by *L. monocytogenes* might differ between strains with different MICs and even between resistant strains with similar MICs but different genetic determinants of BAC resistance. For BAC-R strains similar to S10-1, subminimal inhibitory BAC may represent an advantage, compensating for the weak biofilm formation level that might be associated with resistance. Biofilm formation in the presence of increased subminimal inhibitory concentrations of the disinfectant may represent an important attribute among certain resistant and persistent strains of *L. monocytogenes*.

1. Introduction

The survival and persistence of *Listeria monocytogenes* after cleaning and disinfection in food processing plants represent a major food safety concern (Ferreira et al., 2014). *L. monocytogenes* strains vary in their ability to persist within food environments (Holah et al., 2002; Keto-Timonen et al., 2007; López et al., 2008; Lundén et al., 2003); however, the factors governing persistence in *L. monocytogenes* remain unclear (Carpentier and Cerf, 2011).

The ability to produce biofilms has been investigated as a characteristic favouring the persistence of *L. monocytogenes* strains (Møretrø and Langsrud, 2004). However, the ability of *L. monocytogenes* to form true biofilms is not clear (Ferreira et al., 2014), as the more common method used to quantify *L. monocytogenes* biofilm formation (crystal violet staining of adhered cells) simply reflects cell adherence to a surface rather than a true biofilm (Ferreira et al., 2014). Few studies have employed techniques for characterisation of biofilm structure and architecture, e.g. microscopic methods, and analytical methods for the investigation of extracellular polymeric substances (EPS), e.g. carbohydrate-binding dyes. In addition, biofilm formation assays are difficult to correlate with persistence, most likely due to the influence on biofilm formation of the different experimental conditions tested (Folsom et al., 2006; Nilsson et al., 2011; Pan et al., 2010) and of the different strains assayed in different reports (Djordjevic et al., 2002; Folsom et al., 2006).

In spite of this variation, biofilms provide many outstanding characteristics that enable bacteria to resist harmful environmental conditions, including disinfectants (Bridier et al., 2011; Møretrø and Langsrud,

Abbreviations: BAC, benzalkonium chloride; BAC-R, BAC-resistant strains (MIC ≥ 10 mg/L); BAC-S, BAC-sensitive strains (MIC ≤ 2.5 mg/L); MIC, minimal inhibitory concentration.

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2004). However, resistance to disinfectants of cells grown in biofilms and the resistance of planktonic cells are different phenomena (Kastbjerg and Gram, 2009; Pan et al., 2006; Stopforth et al., 2002). The resistance of planktonic cells might be dependent on intrinsic attributes of the cells, such as the minimal inhibitory concentration (MIC) value of the strain. In several reports, no association between increased MICs of disinfectants and persistence in strains of *L. monocytogenes* have been demonstrated (Earnshaw and Lawrence, 1998; Heir et al., 2004; Holah et al., 2002; Kastbjerg and Gram, 2009; Lourenço et al., 2009).

Disinfectants commonly used in the food industries include quaternary ammonium compounds (QACs), the most common of which is benzalkonium chloride (BAC). The presence of intrinsic and acquired QAC resistance in several foodborne pathogens may result in reduced efficacy of that disinfectant class (Buffet-Bataillon et al., 2012). For *L. monocytogenes*, resistance to BAC has been observed in different countries (Aase et al., 2000; Mereghetti et al., 2000; Mullapudi et al., 2008; Müller et al., 2013; Ortiz et al., 2014b; To et al., 2002). Although the final MIC values of these resistant strains are substantially lower than the concentrations at which QACs are used in food production facilities (Kastbjerg and Gram, 2012), a similar low level of resistance to QACs has been associated with the environmental persistence of *L. monocytogenes* in different food supply chains (Aase et al., 2000; Fox et al., 2011; Lundén et al., 2003; Ortiz et al., 2014b). In addition, it has been observed that *L. monocytogenes* strains which express increased resistance to BAC are generally less susceptible to antibiotics due to overexpression of different multidrug efflux pumps (Rakic-Martinez et al., 2011; Romanova et al., 2006). However, the resistance to BAC does not necessarily imply increased resistance to antibiotics at a level that could be relevant for clinical practice (Ortiz et al., 2014a).

According to Carpentier and Cerf (2011), the presence of growth niches is the main factor that allows for the establishment of *L. monocytogenes* persistence; nevertheless, some strains may possess certain characteristics that increase their chances of becoming persistent (Ferreira et al., 2014). One of these properties might be biofilm formation in the presence of increased subminimal inhibitory concentrations (subMICs) of disinfectants by disinfectant-resistant strains (Nilsson et al., 2011), a foreseeable scenario in certain locations of the food plants (Carpentier and Cerf, 2011).

More specifically, QACs are considered to have poor biodegradability, meaning that contact between bacteria and QACs may be prolonged and, as a consequence, microbial communities are exposed to subinhibitory concentrations (Buffet-Bataillon et al., 2012; Tezel and Pavlostathis, 2012). Subinhibitory concentrations of QACs can change biofilm morphology and architecture (Dynes et al., 2009), and even the effect of QACs at subinhibitory doses on biofilm formation can be stimulatory (Houari and Di Martino, 2007). These effects of subinhibitory QAC on biofilm formation could be different in QAC-resistant or -susceptible strains, similar to the different effect of subinhibitory BAC on the virulence of *L. monocytogenes* observed in strains with different susceptibilities to BAC (Pricope et al., 2013).

Thus, the current study reports the influence of both subminimal inhibitory concentrations of BAC and the MIC values of the strain on biofilm formation by *L. monocytogenes*. We hypothesised that the exposure to subminimal inhibitory concentrations of BAC would have distinct effects on biofilm formation by BAC-resistant strains (BAC-R, MIC $\geq$ 10 mg/L) and BAC-sensitive strains (BAC-S, MIC $\leq$ 2.5 mg/L).

2. Materials and methods

2.1. Strains isolated from the Iberian pork processing plant

Using pulsed-field gel electrophoresis (PFGE), 29 PFGE types were previously identified at an Iberian pork processing plant during a three-year period (Ortiz et al., 2010). The PFGE typing results were analysed in accordance with the optimised PulseNet standardised protocol (Halpin et al., 2010). For the molecular serotyping of isolates of *L. monocytogenes*, a multiplex PCR assay was performed as described by Doumith et al. (2004). The assay differentiated the most common disease-associated conventional serotypes (1/2a, 1/2b, 1/2c, and 4b) into four distinct molecular serotypes. An additional PCR assay based on amplification of the *flaA* gene allowed 1/2a (and 3a) strains to be confirmed and differentiated from atypical 1/2a, 3a, and 1/2c strains (Kérouanton et al., 2010).

After the three-year study, the isolation rate of *L. monocytogenes* decreased, and only four of the 29 PFGE types were found in the following year (Ortiz et al., 2014b). These four "surviving" types included three from molecular serotype 1/2a, which were the only BAC-R subtypes found in the plant (Ortiz et al., 2014b).

The first isolate with a unique PFGE type was considered the PFGE type strain of each PFGE type. In the present study, a subset of 11 PFGE type strains, which represented all of the serotype 1/2a PFGE types (three BAC-R and eight BAC-S), was selected for the characterisation of the biofilm formation. Only one molecular serotype was selected to assess the differences among strains, as there have been some reports of a correlation between serotype and the ability to form biofilms (Borucki et al., 2003; Nilsson et al., 2011; Pan et al., 2010).

2.2. Additional strains of L. monocytogenes

L. monocytogenes EGD-e (ATCC BAA-679), which is a wild-type serotype 1/2a strain, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and included in all tests as a BAC-S reference strain. A BAC-R laboratory mutant ($S2^{BAC}$) of BAC-S strain S2 (Table 1) was obtained by selecting colonies grown on MIC plates inoculated with high inocula (10^9 CFU/mL, ca. 2×10^6 CFU per spot) after an incubation for 48 h at 37 °C. The resistant subculture was prepared from the plate containing the highest concentration of BAC, which resulted in a stable increase in MIC after subculture without BAC. The following strains of *L. monocytogenes* of serotype 1/2a were used as positive controls in the PCR screening of genetic determinants of BAC resistance: 4423 (*qacH* from Tn*6188* and the flanking *radC* gene) and CDL 69 (*bcrABC*) (Müller et al., 2013).

2.3. Standard culture methods and inoculum preparation

Tryptic soy yeast extract broth (TSYEB) and tryptic soy yeast extract agar (TSYEA), both obtained from Biolife (Milano, Italy), were used for routine growth of the strains. A fixed inoculum was prepared and used in all the experiments. Briefly, single colonies from fresh agar plates were transferred to sterile 0.1% (w/v) peptone water (PW, Biolife). The bacterial suspension was adjusted to an absorbance at 600 nm ($A_{600\ nm}$) of 0.85 (corresponding to 10^9 CFU/mL, as determined from a standard measurement relating $A_{600\ nm}$ to plate counts).

2.4. Biofilm formation assay

The assessment of biofilm formation by *L. monocytogenes* was performed using a microplate assay with crystal violet (CV, Sigma-Aldrich, St. Louis, MO, USA) staining and using the $A_{580\ nm}$ reading of destained CV (Pan et al., 2010). Inoculum and polystyrene microtitre plates (Greiner Bio-One, Frickenhausen, Germany) were employed as described by Pan et al. (2010). The experimental culture medium (TSYEB supplemented with 1% glucose and 2% sodium chloride, s-TSYEB) and temperature (37 °C) conditions were selected based on a previous study (Pan et al., 2010) of the optimal conditions for biofilm formation in *L. monocytogenes* of serotype 1/2a. The incubation time was extended from two days (Pan et al., 2010; Ortiz et al., 2014b) to seven days based on findings from preliminary experimentation to address the assay's variability due to low absorbance measurements. Biofilm production was also assessed in a s-TSYEB medium with different concentrations of BAC (from 0.07 to 40 mg/L). For each strain, six wells (rows) of each concentration of BAC (columns) were inoculated.