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Short communication

Industrial disinfectants do not select for resistance in *Listeria monocytogenes* following long term exposure

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ABSTRACT

Listeria monocytogenes is a food-borne pathogen that can persist for years in food processing plants. It has been hypothesized that this could be due to the development of tolerance or resistance to the disinfectants used. The purpose of the present study was to determine whether biocide resistance or tolerance would evolve in *L. monocytogenes* under continued selection in three industrial disinfectants. *L. monocytogenes* EGD was exposed to Desinfect CL (hypochlorite) and Incimaxx DES (peracedic acid and hydrogen peroxide) for several hundred generations. This caused no increase in the minimal inhibitory concentration (MIC) to the disinfectants, whereas exposure to Triquart SUPER (quaternary ammonium compounds) caused a two- to four-fold increase in MIC. Exposure to gentamicin, which was used as a positive control, caused an 8 to 256-fold increase in MIC for several aminoglycosides. Despite the low level of tolerance, the populations adapted to Triquart SUPER were still sensitive to killing with this disinfectant at 0.0125%, which is much lower than in-use concentrations (1–5%). Our data are in agreement with the fact that finding strains with high acquired resistance to disinfectants is rare, and that the disinfectants are still efficient for controlling microorganisms such as *L. monocytogenes*.

1. Introduction

Efficient chemical disinfectants have been invaluable for society for more than 100 years and have provided us with numerous benefits by enabling control of harmful microorganisms in many areas including clinical practice and industrial settings. Even though the disinfectants have been used for such a long time, development of strains with high, acquired resistance to disinfectants at in-use concentrations is seldom reported (Meyer and Cookson, 2010). This is in contrast to antibiotics, where resistance often appears within a few years of their introduction (Palumbi, 2001). This difference in selection for resistance, which we define as a hereditary several fold change in MIC, is believed to be due to several factors such as the differences in the number of targets, as a disinfectant has several targets whereas an antibiotic has one specific target, and also due to the different in-use concentrations. Typically, disinfectants are used in high concentrations e.g. 1000 μg/ml for quaternary ammonium compounds (Meyer, 2006) as compared to the antibiotics, which are used in concentrations near the minimal inhibitory concentration (MIC). However, the trend towards a green profile and the indiscriminate incorporation of biocides (i.e. chemical substances with unspecific antimicrobial action) in many different materials are developments that will cause bacteria to be exposed to lower concentrations of biocides that potentially could lead to increased tolerance, i.e. survival without expressing resistant mechanisms, or actual resistance.

Listeria monocytogenes is a food borne, human pathogen that can cause listeriosis. It is a ubiquitous environmental bacterium and is therefore likely continuously introduced to the different environments were biocides are used. In food-processing plants, some molecular subtypes are able to persist despite cleaning and disinfection (Holah et al., 2002; Keto-Timonen et al., 2007; Miettinen et al., 1999; Wulff et al., 2006). It is likely that such persistent sub-types are exposed to sub-lethal biocide concentrations, and their persistence could potentially be explained by a higher tolerance to biocides. However, results from studies are conflicting and do not point to a clear difference in tolerance to biocides between persistent and non-persistent subtypes of *L. monocytogenes* (Aase et al., 2000; Earnshaw and Lawrence, 1998; Fox et al., 2011; Heir et al., 2004; Holah et al., 2002; Kastbjerg and Gram, 2009). However, long time exposure to sub-inhibitory concentrations could potentially cause an adaptation to disinfectants and evolution of resistance as has been seen for e.g. antimicrobial peptides (Perron et al., 2006).

Others have tried to adapt *L. monocytogenes* to disinfectants in laboratory settings. These studies have not only focused on benzalkonium chloride, which is a quaternary ammonium compound (Aase et al., 2000; To et al., 2002), but have also used industrial disinfectants (Lundén et al., 2003). In these studies, the adaptation procedure is not well described in terms of exposure protocol and the reports seldom state the subsequent highest biocide concentration allowing bacterial growth. Furthermore, the concentration of the disinfectants is increased

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as soon as growth is observed, hence not allowing a longer adaptation at the concentration tolerated. We speculate that a too short adaptation period and a too rapid transfer do not allow the strains to adapt. Typically, two strains with different sensitivities have been used in the studies, and the adaptive response has been greatest when the initial MIC for the strain was low (Aase et al., 2000; Lundén et al., 2003; To et al., 2002). In general, the MIC increased up to 5–6 fold, which typically was the level of the initial MIC of the strain with the highest MIC.

The purpose of this study was to determine whether resistance or tolerance to biocides could evolve in a strain of *L. monocytogenes* that likely has never been exposed to industrial biocides. We used a continued selection in three industrial disinfectants adapting the protocol from Perron et al. (2006) by which a high level of heritable resistance to a cationic antimicrobial peptide evolved. We also included gentamicin, an antibiotic, in the adaptation experiment as a positive control for the protocol since we would expect resistance to evolve faster for this compound as compared to the disinfectants.

2. Materials and methods

2.1. Sequential growth of L. monocytogenes EGD in biocides and gentamicin

L. monocytogenes EGD was grown with three different industrial disinfectants containing different active ingredients: Triquart SUPER (Ecolab, Valby, DK) contains quaternary ammonium compounds, Incimaxx DES (Ecolab, Valby, DK) contains peracetic acid and hydrogen peroxide, and Desinfect CL (Novadan, Kolding, DK) contains hypochlorite. Also, growth in gentamicin (Sigma-Aldrich catalog no. G3632) was included as positive control for the adaptation procedure. All antimicrobial agents were diluted in sterilized, demineralized water. The adaptation was performed with a set-up modified from Perron et al. (2006). One colony of *L. monocytogenes* EGD was grown in brain heart infusion (BHI, Oxoid; CM1135) at 37 °C, 300 rpm overnight (ON), and 30 μl of the outgrown culture was transferred into 27 tubes with 3 ml BHI and incubated ON (37 °C, 300 rpm) giving 27 selection lines (i.e. 27 populations). The selection lines were re-inoculated by transferring 30 μl of stationary phase culture into 3 ml BHI permitting 6–7 doublings before stationary phase. All lines were initially grown in BHI without antimicrobial agents for five transfers. At the sixth transfer, the antibacterial agents were added to BHI in a sub-lethal concentration allowing the selection lines to reach stationary phase in 8 h. Hence, each of the six selection lines was exposed to 0.25% (v/v) Desinfect CL, 0.0625% (v/v) Incimaxx DES or 0.0006% (v/v) Triquart SUPER, respectively, four selection lines were exposed to 0.25 μg/ml GEN and five to sterilized, demineralized water as negative control. Afterwards, we doubled the concentration of the antimicrobial agent every 10 transfers or fewer if the selection line grew rapidly to high turbidity (Fig. 1). If the selection line was not able to grow in the increased concentration it was transferred to the previous lower concentration. Every time the concentration of the antibacterial agent was increased, 700 μl of the ON culture was transferred to 300 μl 50% (v/v) glycerol and stored at −80 °C. The experiment was conducted for approx. 50–60 serial transfers, giving a total of 300–420 bacterial generations. All selection lines were streaked on BHI-agar (BHI with 1.5% agar (VWR catalog no. 20768.292)) daily to check the purity and occasionally verified as *Listeria* by cultivation on *Listeria* selective agar base (Oxoid catalog no. CM0856) supplemented with modified *Listeria* selective supplement (Oxoid catalog no. SR0206E) and as *L. monocytogenes* by cultivation on RAPID'L.Mono (Bio-rad catalog no. 3563694).

2.2. Determination of susceptibility of adapted populations

To determine if the susceptibility had changed during the adaptation, the minimal inhibitory concentration (MIC) was determined for each selection line using the population kept from the highest concentration of the antimicrobial agent with growth. 42 μl of the freeze culture was inoculated into 3 ml BHI containing the compound concentration from which the population was isolated, incubated ON (37 °C, 300 rpm), diluted 100× and re-grown ON in BHI with compound. MIC was determined by the use of a microbroth dilution method with modifications (Clinical and Laboratory Standards Institute, 2006). Two-fold dilution series were made of the following antibacterial agents in BHI broth in microtiter plates (Nunc A/S, 163320): gentamicin, kanamycin (Sigma-Aldrich, catalog no. K4000), streptomycin (Sigma-Aldrich, catalog no. S6501), tobramycin (Sigma-Aldrich, catalog no. T1783), Incimaxx DES, Desinfect CL and Triquart SUPER. The MIC was determined after 24 h at 37 °C using a starting concentration of approx. $5 \cdot 10^5$ CFU/ml of each selection line in the microtiter plate. Cultures from 1/2× MIC were streaked on BHI agar to check the purity. The MIC was determined in at least biological duplicates for each selection line.

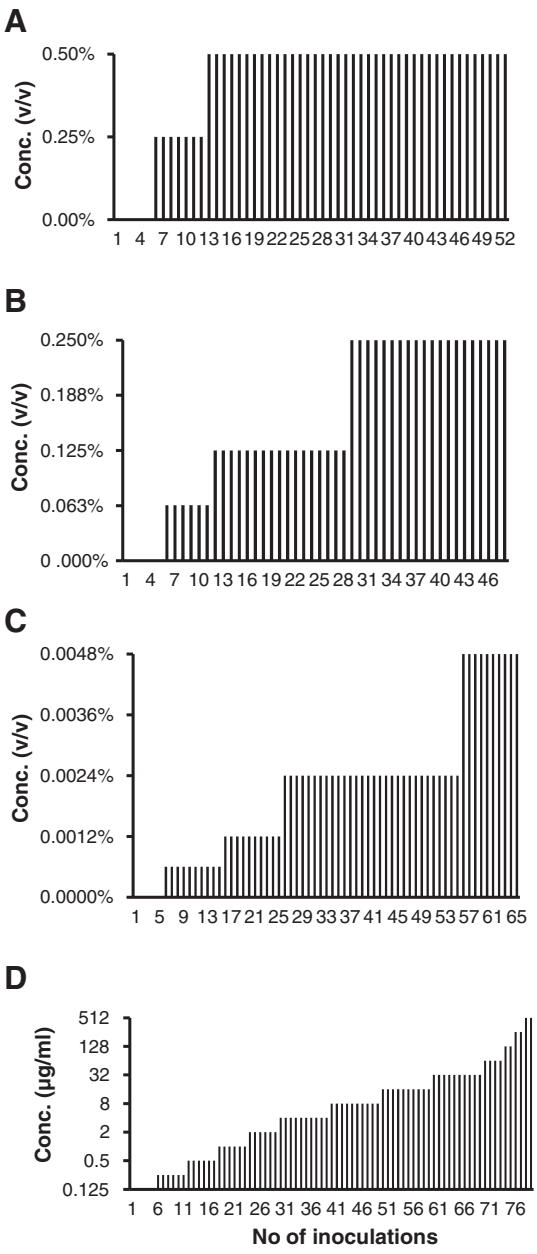

Fig. 1. Increase in concentration of compounds used for adaptation of *Listeria monocytogenes* EGD to (A) Desinfect CL, (B) Incimaxx DES, (C) Triquart SUPER, (D) gentamicin. The procedure presented is representative for the adaptation of the selection lines, six for each of the disinfectants and four for GEN, respectively.