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Effects of exposure to quaternary-ammonium-based biocides on antimicrobial susceptibility and tolerance to physical stresses in bacteria from organic foods

Rebeca Gadea, Miguel Ángel Fernández Fuentes, Rubén Pérez Pulido, Antonio Gálvez*, Elena Ortega

Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071, Jaén, Spain

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ABSTRACT

In the present study, a collection of 76 biocide-sensitive bacterial strains isolated from organically produced food were adapted by repeated exposure to increasing concentrations of the quaternary ammonium compounds (QACs) benzalkonium chloride (BC) and hexadecylpyridinium chloride (HDP). The sensitivity of both wildtype strains and their corresponding QAC-adapted strains to other biocides and to antibiotics was studied. QAC tolerance increased in 88.2% of strains for BC and in 30.3% of strains for HDP, with increases in minimum inhibitory concentrations between 2 and over 100 fold. Adaptive resistance was stable after 20 subcultures in biocide-free medium for 7 and 5 of the BC- and HDP-adapted strains, respectively. Adaptation to BC and HDP also reduced the susceptibility to other biocides, mainly hexachlorophene (CF), didecyldimethylammonium bromide (AB), triclosan (TC) and chlorhexidine (CH). BC-adapted strains showed increased antibiotic resistance to ampicillin (AM) followed by sulfamethoxazol (SXT) and cefotaxime (CTX), and some showed increased sensitivity to ceftazidime (CAZ), CTX, AM and STX. Changes in antibiotic resistance in HDP-adapted strains were more heterogeneous and strain-dependent. Main efflux pump genes detected in QAC-adapted strains were *acrB*, *sugE*, *norC*, *qacE* and *qacH*, as well as antibiotic resistance genes *aac(6_)-Ie-aph(2_)-Ia, aph(2_)-Ic, ant(4_)-Ia, lsa, mrsA/B, ereA, ermB* and *cat*. Membrane anisotropy experiments revealed that QAC adaptation induced an increase in membrane rigidity in the case of BC, while response to HDP was more heterogeneous and strain-dependent. Growth capacity was significantly higher in some QAC-adapted strains and strain-dependent changes in heat tolerance were also detected in QAC-adapted strains. Gastric acid or bile resistances do not seem to be influenced by QAC adaptation.

1. Introduction

Disinfectants play an important role in maintaining acceptable health standards by significantly reducing microbial loads as well as inactivating pathogens. Quaternary ammonium compounds (QACs) are cationic surfactants introduced in the late 1930s and mainly used in disinfectant and antiseptic formulations utilized in human and animal healthcare facilities, agriculture and industry. Currently, QACs are the major class of cationic surfactants used as the ingredients in fabric softeners, disinfectants, biocides, detergents, phase transfer agent and numerous personal care products. They are effective against a variety of bacteria, fungi and viruses at very low concentrations. However, inappropriate application of disinfectants, dilution in the environment after discharge and biodegradation result in biocide concentration gradients. Moreover, because QACs are biodegradable under aerobic conditions, their concentrations in indoor and outdoor environments continuously fluctuate. As a result, microorganisms are exposed to QACs dynamically over a wide range of concentrations (i.e., non-inhibitory, sub-inhibitory, over-inhibitory concentrations) (Tezel and Pavlostathis, 2015). Recent studies suggest that exposure to sub-inhibitory biocide concentrations facilitates the evolution of resistance to the biocide, and may also lead to co-resistance and cross-resistance to other antimicrobial agents such as antibiotics

* Corresponding author. Present address: Área de Microbiología, Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Edif. B3, Universidad de Jaén, Campus Las Lagunillas s/n, 23071, Jaén, Spain.
E-mail address: agalvez@ujaen.es (A. Gálvez).

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(Mc Cay et al., 2010; Elhanafi et al., 2010; Hegstad et al., 2010; Rakic-Martinez et al., 2011). A certain antimicrobial agent might directly select for resistance directed against itself, might indirectly (cross-) select for cross-resistance to chemically related agents and/or might (co-)select for co-resistance to unrelated substances (Shah, 2005).

Because many of the QAC resistance pathways and mechanisms are similar to those involved in antibiotic resistance, understanding QAC resistance and dissemination is very important in the context of the global antibiotic resistance problem. Although some suggest that there is no clear relationship between antibiotic resistance and exposure of microorganisms to QACs (Gerba, 2015), many studies have shown that exposure to QACs results in dissemination of resistance genes (Gillings et al., 2015; Oh et al., 2013; Zou et al., 2014).

The mode of action of QACs at sub-MICs is complicated and always includes multiple processes such as loss of membrane osmoregulation, inhibition of respiratory enzymes, the dissipation of proton motive force and oxidative stress (Blazquez et al., 2012; Ceragioli et al., 2010). The most widespread mechanism leading to decreased susceptibility to QACs is increased efflux pump activity (Poole, 2005) although other mechanisms may be involved such as altered fatty acid composition and changes in the bacterial membrane (Ferreira et al., 2011; Guerin-Mechin et al., 2000). Indeed, multiple mechanisms co-develop during adaptation of bacteria to QACs, as modification of cell membrane structure and composition, enhanced biofilm formation, acquisition of efflux genes, overexpression of efflux pump systems, and biodegradation (Moen et al., 2012).

The aim of the present study was to analyze the effects of step-wise exposure of biocide-sensitive bacteria isolated from organic foods to the QACs benzalkonium chloride and hexadecylpyridinium chloride. The analysis included changes in the tolerance to the biocide itself, the tolerance to other biocides, and cross-resistance to clinically important antibiotics. The involvement of efflux mechanisms was studied on the basis of the presence of multidrug efflux pumps genes and restored sensitivity of Gram-positive strains in the presence of the efflux pump inhibitor (EPI) reserpine. Anisotropy measurements in wildtype and biocide-adapted strains were used to analyze the effect of step-wise exposure to QACs on membrane fluidity and its implication in the resistance mechanisms.

In order to determine the effect of biocide-induced sub-lethal injury of bacteria on subsequent resistance to other stresses, as physical treatments usually applied in food industry, as well as on the physiological protection against food-borne pathogens along the host gastrointestinal tract, strains were investigated for growth capacity and resistance to heat, gastric acid and bile salts.

2. Material and methods

2.1. Bacterial strains

A total of 76 biocide-sensitive bacterial strains previously isolated from organic foods and classified as sensitive to biocides and antibiotics (Fernández-Fuentes et al., 2012) were selected for this study. Strains were isolated from samples of 39 commercial organic foods (including flours, fruits and vegetables, legumes, cereals, rice, pastes, sauces, cheeses and manufactured products). All studied foods were certificated as obtained by organic production according to Spanish regulations. Strains were stored at −80 °C in Brain Heart Infusion (BHI) broth (Scharlab, Barcelona, Spain) supplemented with 20% glycerol. For the preparation of inocula, strains were incubated for 15 h in BHI broth at 37 °C.

2.2. Strain identification

Strains were identified by conventional tests (Gram staining, catalase and oxidase tests) and 16S rDNA sequencing in a previous study (Gadea et al., 2016) or in the present study, as specified in Tables 1 and 3 DNA was extracted with a bacterial genomic DNA extraction kit (GenElute™, Sigma-Aldrich, Madrid) and 16S rDNA was amplified as described by Abriouel et al. (2005). PCR amplification products were purified using a GFX PCR DNA and Gel Band Purification Kit (GE-Healthcare, Spain), and then sequenced by using the primers Sp3 (5′-TACGCATTTCACCKCTACA-3′, position 684 reverse), Sp4 (5′-CTCGTTGCGGGACTTAAC-3′, position 1089 reverse) and Sp5 (5′-GNTACCTTGTTACGACTT-3′, position 1492 reverse) according to Weisburg et al. (1991) in a CEQ 2000 XL DNA Analysis System (Beckman Coulter, CA, USA). The DNA sequence of amplicons was determined by using CEQ 2000 dye terminator cycle sequencing with Quick Start kit (Beckman Coulter, CA, USA) according to the manufacturer's instructions. The sequence data were analyzed with a CEQ DNA analysis system (version 4.0). The overlapping sequences obtained with SP3, SP4 and SP5 were merged using Lasergene programme (DNASTAR, Inc., Madison, WI, USA). A search for homology of the DNA sequence was done using the BLAST algorithm available at the National Centre for Biotechnology Information (NCBI, USA).

2.3. Adaptation to biocides

The tolerance of sensitive strains was gradually increased by serially inoculating 100 μl from an overnight bacterial culture into 10 ml of Trypticase Soya Broth (TSB; Scharlab) containing a range of concentrations of the quaternary-ammonium-based biocides benzalkonium chloride (BC) and hexadecylpyridinium chloride (HDP), according to the technique described by To et al. (2002). The cultures were incubated at 37 °C for 24–48 h and additional subcultures were prepared from the tube containing the highest concentration of biocide that resulted in turbidity after incubation. Strains were subcultured in tubes containing the same concentration and the next higher concentration of biocides. This procedure was continued until no growth was observed after 72 h of incubation at 37 °C. The concentrations of the biocides were as follows: 0.01, 0.1, 1, 5, 10, 50, 100, 200, 500 μg/ml, 1, 2, 5 and 10 mg/ml, depending upon the growth of the adapted microorganism. Control strains were cultivated in biocide-free medium in parallel to adapted strains. The suspension in the last tube with recorded growth was seeded on a TSA plate and the bacterial growth was collected, resuspended in 1 ml BHI broth (Scharlab) supplemented with 20% glycerol and stored at −80 °C. The stability of the adaptive tolerance was determined in each adapted strain by repeated subculture in biocide-free medium. Subcultures were performed every 24 h, for 20 days. The MICs were determined after 5, 10, 15 and 20 passages.

2.4. Determination of adaptive tolerance, sensitivity to biocides and antibiotics

The wildtype strains and the corresponding strains adapted to BC and HDP (67 and 23 of the 76 strains studied, respectively) were tested for sensitivity to other biocides and to antibiotics. The minimum inhibitory concentrations (MICs) of biocides and antibiotics were determined by the broth microdilution method in 96-well microtiter plates. Briefly, serial dilutions of each substance (previously dissolved in absolute alcohol when necessary) were incubated with bacterial suspensions adjusted to 5×10^5 colony-forming units (CFU)/ml in TSB (Scharlab). Growth and sterility controls were included for each isolate, as well as the vehicle, as a