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Adaptive tolerance to phenolic biocides in bacteria from organic foods: Effects on antimicrobial susceptibility and tolerance to physical stresses

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ABSTRACT

The aim of the present study was to analyze the effects of step-wise exposure of biocide-sensitive bacteria from organic foods to phenolic biocides triclosan (TC) and hexachlorophene [2,2′-methylenebis(3,4,6-trichlorophenol)] (CF). 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 also studied as well as the possible implication of modifications in cytoplasmic membrane fluidity in the resistance mechanisms. The influence of biocide tolerance on growth capacity of the adapted strains and on subsequent resistance to other physical stresses has also been analyzed. Repeated exposure of bacteria from organic foods to phenolic biocides resulted in most cases in partially increased tolerance to the same biocide, to dissimilar biocides and other antimicrobial compounds. Nine TC-adapted strains and six CF-adapted strains were able to develop high levels of biocide tolerance, and these were stable in the absence of biocide selective pressure. Most strains adapted to TC and one CF-adapted strain showed significantly higher anisotropy values than their corresponding wildtype strains, suggesting that changes in membrane fluidity could be involved in biocide adaptation. Exposure to gradually increasing concentrations of CF induced a decrease in heat tolerance. Biocide adaptation had no significant effects of gastric acid or bile resistance, suggesting that biocide adaptation should not influence survival in the gastrointestinal tract.

1. Introduction

Biocides are widely used in health and agricultural settings as well as in the food industry to prevent bacterial contamination. However, elimination of food-borne bacteria has proven difficult and it has become clear that the efficacy of biocides may be questionable in some circumstances (Davidson & Harrison, 2002). Under-dosing of applied disinfectants and insufficient cleaning before disinfection can also significantly reduce the efficacy of disinfectants. Under such conditions, bacteria are regularly exposed to sub-lethal concentrations of disinfectants, and this can lead to adaption and reduction of susceptibility to dissimilar disinfectants or antimicrobial compounds (Braoudaki & Hilton, 2005; Loughlin, Jones, & Lambert, 2002; Tattawasart, Maillard, Furr, & Russell, 1999; Thomas, Maillard, Lambert, & Russell, 2000). Moreover, it has been suggested that exposure to sub-lethal concentrations of biocides could potentially have an impact on the responses of bacteria to commonly used food processes, enabling microorganisms to survive challenges such as the concentrations of biocides currently permitted for use in food environments (Sheridan, Lenahan, Duffy, Fanning, & Burgess, 2012) or different physical and chemical food treatments. However, at present, there is a limited understanding of the mechanisms which contribute to biocide tolerance.

Phenols, cresols, and their chlorinated derivatives may not only act on several cellular targets, but also induce leakage of intracellular materials from bacteria (McDonnell & Russell, 1999). At low concentrations, triclosan inhibits the NADH-dependent enoyl-[acyl carrier protein] reductase (Schweizer, 2001) but at higher concentrations, it also has membranotropic effects (Villalain, Mateo, Aranda, Shapiro, & Micol, 2001). This is in contrast to antibiotics, which are considered to have only one major target site of activity (Russell, 2003) thereby, facilitating the development of resistance. However, recent evidence suggests that mechanisms providing tolerance to biocides may also provide cross-protection to the activity of antibiotics (Condell et al., 2012; Mavri & Smole Možina, 2013; Middleton & Salierno, 2013). It has been suggested that cross-resistance to antimicrobial compounds, following exposure and adaptation to a biocide, could occur in a limited number of situations. These can be summarized as follows: (i) when the biocide and an antimicrobial compound act on the same cellular target, (ii) when the biocide and the antimicrobial compound have the same transport mechanism, (iii) where a biocide and antibiotic can be accommodated by the same resistance mechanism (Gilbert & McBain, 2003), and finally, (iv) in situations where genes contributing toward biocide tolerance and antibiotic resistance are carried on the same mobile genetic element

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(Poole, 2004). Exposure to biocides in the environment may select for bacterial strains tolerant to these compounds and exhibiting increased resistance to antibiotics through co- or cross-resistance mechanisms (Chuanchuen et al., 2001; Maillard, 2007; Cottell, Denyer, Hanlon, Ochs, & Maillard, 2009).

There is also a need to determine the effect of biocide-induced sub-lethal injury of bacteria on subsequent resistance to other stresses. Cross-protection against heat as a result of various environmental stresses, including exposure to starvation conditions, ethanol, acids, H_2O_2 and alkali has been previously described in different bacteria (Leyer & Johnson, 1993; Lou & Yousef, 1996; Ryu, Deng, & Beuchat, 1999). Gastrointestinal tract (GIT) stress factors also influence the ability of food-borne bacteria to survive and maintain their effects on the host. Biocide adaptation of bacterial cells could be responsible for subsequent increased tolerance to both physical treatments usually applied in food industry and physiological protection against food-borne pathogens along the host gastrointestinal tract. These aspects should be further investigated.

The aim of the present study was to analyze the effects of step-wise exposure of biocide-sensitive bacteria from organic foods to phenolic biocides triclosan (TC) and hexachlorophene [2,2′-methylenebis(3,4,6-trichlorophenol)] (CF). The analysis included changes in the susceptibility to the biocide itself, the susceptibility to other biocides, and cross-resistance to clinically important antibiotics. The involvement of efflux mechanisms was studied on the basis of presence of multidrug efflux pumps genes and restored sensitivity in the presence of the efflux pump inhibitor (EPI) reserpine in Gram-positive strains. Anisotropy values of the fluorescence polarization of DPH (1,6-diphenyl-1,3,5-hexatriene) in wildtype and biocide-adapted strains were analyzed in order to determine the influence of exposure to increasing sub-lethal concentrations of these biocides on membrane fluidity. The effect of biocide-induced sub-lethal injury of bacteria on growth capacity and on subsequent resistance to other physical stresses as heat, gastric acid and bile salts was also analyzed.

2. Materials 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, Ortega Morente, Abriouel, Pérez Pulido, & Gálvez, 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

In the present study, the selected strains were identified by conventional tests (Gram staining, catalase and oxidase tests) and 16S rDNA sequencing. 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, Barns, Pelletier, and Lane (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 program, version 5.05 (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 Tryptic Soy Broth (TSB; Scharlab, Barcelona) containing a range of concentrations of the phenolic biocides triclosan (TC) and hexachlorophene [2,2′-methylenebis(3,4,6-trichlorophenol)] (CF) according to the technique described by To, Favrin, Romanova, and Griffiths (2002). The cultures were incubated at 37 °C for 24 to 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 supplemented with 20% glycerol and stored at −80 °C.

The stability of the biocide-tolerant phenotype 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. The culture purities were analyzed at each stage in terms of cell morphology and Gram staining.

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

The wildtype strains and the corresponding strains adapted to TC and CF 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 Trypticase Soya Broth (TSB; Scharlab). Growth and sterility controls were included for each isolate, as well as the vehicle, as a negative control. Microtiter plates were incubated at 37 °C and readings were performed after 20 h of incubation by visual reading and optical density (OD 595 nm) determination in an iMark Microplate Reader (BioRad, Madrid). The MIC value was defined as the lowest compound concentration that prevented bacterial growth after incubation for 20 h. When turbidity of wells did not allow OD to be determined, counts of CFU after plating 100 μl from the wells onto nutrient agar plates were used to determine the minimum bactericidal concentration (MBC).

Biocides employed for the assays – didecyldimethylammonium bromide (AB), cetrimide (CE), hexachlorophene [2,2′-methylenebis(3, 4,6-trichlorophenol)] (CF), chlorhexidine (CH), hexadecylpyridinium chloride (HDP), benzalkonium chloride (BC) and triclosan (TC) – were obtained from Sigma-Aldrich. Antibiotics were selected as representatives of main groups widely used for human therapy. Ampicillin (AM) was obtained from Laboratorio Reig Jofré, Barcelona (Spain), cefotaxime (CTX) and ceftazidime (CAZ) from Laboratorios Normon, Madrid (Spain), ciprofloxacin (CIP) from Fluka, Madrid (Spain), imipenem