Food Control 26 (2012) 98–106

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

The potential for biocide tolerance in *Escherichia coli* and its impact on the response to food processing stresses

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ARTICLE INFO

Article history:
Received 2 August 2011
Received in revised form
21 December 2011
Accepted 3 January 2012

Keywords:
Verocytotoxigenic *E. coli* (VTEC)
Biocide resistance
Antibiotic resistance
Triclosan
Benzalkonium chloride

ABSTRACT

Biocides are used at all stages of the farm-to-fork continuum to reduce or eliminate both pathogens and spoilage microorganisms. Currently there is limited understanding of the mechanisms which contribute to biocide tolerance. Also, the impact of this phenotype may affect other risk reduction measures applied across the food chain. Tolerance to one or more biocides may contribute to an increase in the persistence of foodborne pathogens and other bacteria in the food chain. A panel of verocytotoxigenic and non toxigenic *Escherichia coli* strains were screened for their tolerance to eight commercial biocides and three biocidal active compounds (triclosan, benzalkonium chloride and chlorhexidine). Minimum inhibitory concentrations (MIC) for the commercial biocides were lower than the working concentration recommended by the manufacturers, while the MICs for the biocidal active components ranged from 0.78 to 12.5 μg/ml. As a means of exploring the associated phenotypes, mutant strains were selected which had an increased tolerance to biocides. A stepwise broth method was used to isolate biocide tolerant mutants. No stable mutants could be selected when commercial biocide preparations were used. In contrast, three stable mutants were isolated displaying an increased tolerance to the biocidal active component triclosan with growth observed at >8 mg/ml in comparison to the MIC for the wildtype strain (6.25 μg/ml). One isolate showed an increased tolerance to benzalkonium chloride. The responses of both isogenic wildtype and mutant isolates were compared to stresses often encountered across the food production chain. The strains were exposed to acid at pH 2 and 4, and to temperatures of 55 and 62 °C. No significant differences were observed in the survival of the wildtype and mutant strains under these environmental conditions, except in the case of *E. coli* O103 (T11 2EF1) at 55 °C ($P < 0.001$) and *E. coli* O157 (T3 5H5) at 62 °C ($P < 0.05$).

1. Introduction

Biocides are antimicrobial compounds used as disinfectants, antiseptics or preservatives to reduce or eliminate microbial contamination. Several biocidal agents of different classes are available commercially, such as alcohols, oxidising agents, hypochlorite, quaternary ammonium compounds (QAC), phenolics, triclosan and chlorhexidine (IFT, 2006). Generally, biocidal active compounds act at multiple target sites within the microbial cell causing cellular damage which results in the bactericidal effect (Maillard, 2002). However, other biocidal active agents such as triclosan target the biosynthesis of fatty acids through non-competitive inhibition of the NADH dependent enoyl-acyl carrier protein (ACP) reductase (*fabI*) in *Escherichia coli* (McMurry, Oethinger, & Levy, 1998). Previous work in *E. coli* has suggested that substitutions within FabI are the primary mechanism mediating to triclosan resistance (Sivaraman, Zwahlen, & Bell, 2003). Several biocidal compounds can be combined together to increase their effectiveness, a practice commonly undertaken during the development of commercial biocides.

Biocides are deployed at all stages of the farm-to-fork continuum to reduce or eliminate commensal and pathogenic microorganisms. However, bacteria have been shown to adapt rapidly to environmental conditions and stresses designed to counteract them (Jansen, Van der Bruggen, Verhoef, & Fluit, 2006). Tolerance to a biocide was first recognised almost 75 years ago (Heathman, Pierce, & Kabler, 1936) and in some cases bacterial resistance has emerged following the improper storage of biocides, resulting in a decrease in the effective biocide concentration (Russell, 2002).

Resistance mechanisms contributing to biocidal tolerance often involve changes in cell envelope permeability and altered activity

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doi:10.1016/j.foodcont.2012.01.018

of membrane associated efflux pumps. Changes in cell envelope permeability limit the amount of biocide that can enter the bacterium, thereby reducing the intracellular biocide concentration (Champlin, Ellison, Bullard, & Conrad, 2005; Denyer & Maillard, 2002). Altered activity of efflux pumps can also support resistance by actively pumping a wide range of toxic substrates, including antibiotics and biocides from the cell preventing accumulation of these agents. Efflux pumps contributing to this phenotype in *E. coli* include AcrAB-TolC, AcrEF-TolC and EmrE (Levy, 1992, 2002).

An increase in biocide tolerance is an important public health issue which could be expected to contribute to the increased persistence of pathogens in the food chain. There is limited understanding on the development of biocide resistance in important foodborne pathogens such as verocytotoxigenic *E. coli* (VTEC). *E. coli* O157:H7 is the most prevalent VTEC serotype associated with human infection and has been linked to many outbreaks of food poisoning across the world (Karmali, 2009). Other non-O157 VTEC serotypes, including O26, O103 and O145 are also recognised as important pathogens and are being increasingly linked to human infections (Karch, Bielaszewska, Bitzan, & Schmidt, 1999). In 2011, Germany reported a major *E. coli* O104:H4 outbreak linked with fresh sprouted seeds, where 3910 cases of diarrhoeal disease were reported including 782 cases of haemolytic uraemic syndrome (HUS) and 46 deaths (ECDC, 2011). There is concern that such organisms if biocide resistant may have a reduced susceptibility to clinically important antimicrobial compounds such as antibiotics (Russell, 2002) although there is controversy about antibiotic therapy for VTEC infections (Dundas & Todd, 2000; Panos, Betsi, & Falagas, 2006).

Studies have shown foodborne pathogens such as VTEC can persist in food processing environments and subsequently contaminate foods. In an attempt to overcome this food processing risk, reduction measures have been developed to minimise human and animal exposure to these pathogens. Some of these measures include the use of heat treatments to eliminate microorganisms in food production processes. Low pH food matrices can also be used to control the growth of pathogens (Yuk & Marshall, 2004). These two food preservation techniques can also be combined as a multi-hurdle approach to reduce the persistence of pathogens in foods. Biocide resistance could potentially impact on bacterial responses to commonly used food processes enabling them to survive challenges such as heat and acid pH during food processing.

This study set out to evaluate the level of tolerance among a collection of *E. coli* to commonly used biocides and antimicrobial compounds and to determine whether selected isogenic mutants demonstrated an increased tolerance towards some of the active agents. It also compared the survival of both isogenic wildtype and mutant isolates to low pH and heat stresses commonly encountered in food processing.

2. Material and methods

2.1. Bacterial strains

Bovine VTEC isolates ($n = 99$), including *E. coli* O157 ($n = 53$), O103 ($n = 30$), O26 ($n = 14$) and O145 ($n = 2$), were obtained from a tracking study undertaken in Irish beef abattoirs (Thomas et al., 2011). All O157, O26, O103 and O145 strains were assessed for the presence of the verotoxin genes as well as *eae* and *hlyA* according to the protocol outlined by Paton and Paton (1998). Of the O157 isolates ($n = 53$) nine were positive for *vtx1*, *vxt2*, *eae* and *hlyA*, 41 were positive for *vtx2*, *eae* and *hlyA*, one was positive for *vtx2* and *eae*, one was *vtx2* positive only and one strain contained none of these genes. All of the O26 isolates contained *eae* only, except for one isolate which contained *vtx1*, *eae* and *hlyA* and one strain which contained all four genes. The O145 isolates contained *eae* and *hlyA* only. The O103 isolates mainly contained *eae* and *hlyA* only ($n = 22$). However, one strain contained *vtx1* and *vtx2* only, one strain contained *vtx1*, *eae* and *hlyA*, one strain contained *hlyA* only, two strains contained *eae* only and three strains contained none of these genes (Thomas et al., 2011). As part of that study *XbaI* pulse field gel electrophoresis of a subset of verotoxin positive isolates was undertaken. This included O157 isolates ($n = 25$) used in this study, which showed greater than 86% similarity. Non pathogenic *E. coli* ($n = 7$) from equine sources were obtained from the Veterinary Hospital at University College Dublin. Type strain *E. coli* O157 EDL933 (ATCC®700927D) was included as a control. Bacterial cultures were routinely stored on Protect™ beads (Technical Service Consultants, Lancashire, United Kingdom) and maintained frozen at −20 °C.

2.2. Antibiotic resistance

The resistance profiles of VTEC ($n = 100$) and equine *E. coli* ($n = 7$) isolates were tested using the Sensititre NARMS Gram-negative minimum inhibitory concentration (MIC) plate (TREK Diagnostic Systems, Westlake, Ohio USA). This assay screens for resistance to a panel of 15 different antimicrobial compounds including ampicillin (AMP), amikacin (AMI), amoxicillin/clavulanic acid (AUG) ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), sulfamethoxazole (SXT), cefoxitin (FOX), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), sulfisoxazole (FIS), streptomycin (STR), tetracycline (TET) and ceftiofur (TIO). Susceptible, intermediate or resistant cut offs were defined according to CLSI guidelines (CLSI, 2008). A micro-dilution technique was used to carry out the test according to guidelines set out by the Clinical and Laboratory Standards Institute (CLSI, 2008). Cultures were grown in 11 ml Mueller Hinton broth (MH; Sigma–Aldrich, Munich, Germany) for 18 h at 37 °C and diluted according to the Sensititre manufacturer's instructions to give an inoculation concentration of 10^5 cfu/ml. Aliquots (50 μl) of culture containing 10^5 cfu/ml were dispensed into the Sensititre plate containing the antimicrobial compounds. Plates were incubated statically at 35 °C and growth was checked after 24 h by measuring the absorbance at 600 nm (OD_{600nm}) using a Tecan plate reader (Tecan, Mannedorf, Switzerland).

2.3. Biocide MIC determination

Eight commercially available biocides (denoted as A through H) commonly used in food plants were included in the study (Table 1). VTEC ($n = 100$) and equine *E. coli* ($n = 7$) were assessed for their tolerance to each biocide preparation. In parallel, three biocidal active agents (triclosan, benzalkonium chloride and chlorhexidine; Sigma–Aldrich) were used to screen a subset panel of 20 isolates comprising of *E. coli* O157 ($n = 8$), O103 ($n = 5$) and equine *E. coli* ($n = 7$) (Table 1).

Biocide tolerance was measured for each isolate using a microtitre plate assay to determine the MIC for each of the selected biocides. This assay was performed in sterile 96-well polypropylene plates (Sigma–Aldrich). Biocides were diluted across the rows in serial two-fold dilutions from 0 to 200% of manufacturer's recommended working concentration for the commercial biocides and 0–200 μg/ml for the biocidal components. One Protect™ bead containing each isolate was initially streaked onto Luria-Bertani (LB) agar (Sigma–Aldrich) and incubated for 18 h at 37 °C to recover the test organism. A single colony was removed and placed into a tube containing 10 ml MH broth and incubated for 18 h at 37 °C. The overnight culture was diluted in 10 ml double strength MH broth to give a concentration of 10^5 cfu/ml. Aliquots of the culture (100 μl) were dispensed into the 96-well microtitre plate