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Inactivation strategy for *Clostridium perfringens* spores adhered to food contact surfaces

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ABSTRACT

The contamination of enterotoxigenic *Clostridium perfringens* spores on food contact surfaces posses a serious concern to food industry due to their high resistance to various preservation methods typically applied to control foodborne pathogens. In this study, we aimed to develop an strategy to inactivate *C. perfringens* spores on stainless steel (SS) surfaces by inducing spore germination and killing of germinated spores with commonly used disinfectants. The mixture of L-Asparagine and KCl (AK) induced maximum spore germination for all tested *C. perfringens* food poisoning (FP) and non-foodborne (NFB) isolates. Incubation temperature had a major impact on *C. perfringens* spore germination, with 40 °C induced higher germination than room temperature (RT) (20 ± 2 °C). In spore suspension, the implementation of AK-induced germination step prior to treatment with disinfectants significantly ($p < 0.05$) enhanced the inactivation of spores of FP strain SM101. However, under similar conditions, no significant spore inactivation was observed with NFB strain NB16. Interestingly, while the spores of FP isolates were able to germinate with AK upon their adhesion to SS chips, no significant germination was observed with spores of NFB isolates. Consequently, the incorporation of AK-induced germination step prior to decontamination of SS chips with disinfectants significantly ($p < 0.05$) inactivated the spores of FP isolates. Collectively, our current results showed that triggering spore germination considerably increased sporicidal activity of the commonly used disinfectants against *C. perfringens* FP spores attached to SS chips. These findings should help in developing an effective strategy to inactivate *C. perfringens* spores adhered to food contact surfaces.

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1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium, which can be classified into 5 types, A-E (McClane, 2007). The small group (less than 5%) of *C. perfringens* type A isolates produces *C. perfringens* enterotoxin (CPE), which is responsible for most symptoms of *C. perfringens*-associated gastrointestinal (GI) diseases (Lindström et al., 2011; McClane, 2007; Sarker et al., 1999). CPE encoding gene (*cpe*) can be located either on the chromosome or on large plasmids in *C. perfringens* (Brynestad et al., 1997; Cornillot et al., 1995). The chromosomal *cpe*-positive strains (C-*cpe*) are generally linked to food poisoning (FP) due to its higher resistance to heat, low temperature, NaCl, and nitrite than the plasmid-borne *cpe* carrying strains (P-*cpe*), while the P-*cpe* isolates are associated with non-foodborne (NFB) GI diseases (Collie and McClane, 1998; Li and McClane, 2006a,b; Lindström et al., 2011; Sarker et al., 2000; Sparks et al., 2001). Nevertheless, the recent investigations suggested that P-*cpe* isolates could be a causative agent for *C. perfringens* type A FP (Lahti et al., 2008; Tanaka et al., 2003). Since *C. perfringens* spores are much more resistant than their vegetative counterparts to a variety of lethal factors such as heat, prolonged refrigeration and frozen temperatures, chemicals, and high hydrostatic pressure (Li and McClane, 2006a,b; Paredes-Sabja et al., 2007; Sarker et al., 2000), they can survive thermal processing and sanitizing treatments employed in the food industry. Also, due to the spore's high hydrophobic characteristics, spore elimination is usually more difficult when attached to food contact surfaces (Blatchley et al., 2005). The contaminated food contact surfaces could be the potential source of pathogen transmission to food products in the food processing, catering, and domestic environments (Bae and Lee, 2012; Kusumaningrum et al., 2003).

It is well recognized that germinated spores are more susceptible to various stress factors than their dormant form (Akhtar et al.,

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2009; Clouston and Wills, 1969; Nerandzic and Donskey, 2010); therefore, inducing spore germination prior to disinfection step could be a potential strategy to improve elimination or reduction of *C. perfringens* spores from food contact surfaces. Previous studies demonstrated the use of this strategy in reducing *C. perfringens* spores in poultry products, as well as in increasing sensitivity of *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus cereus*, *Clostridium difficile*, and *Clostridium botulinum* spores to subsequent inactivation by heat, radiation, and chemicals (Akhtar et al., 2009; Durban et al., 1970; Gould et al., 1968; Løvdal et al., 2011; Munakata, 1974; Nerandzic and Donskey, 2010; Stuy, 1956). In the current study, we aimed to develop an inactivation strategy for *C. perfringens* spores attached to stainless steel (SS) surfaces by inducing spore germination followed by subsequent killing of germinated spores with disinfectants typically used in food processing facilities and domestic kitchens. Our results showed that inducing germination significantly increased sporicidal activity of commonly used disinfectants against spores of *C. perfringens* FP isolates on SS surfaces.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The enterotoxigenic *C. perfringens* type A isolates used in this study consists of 5 FP isolates (SM101, NCTC8239, NCTC10239, E13, and 6263) and 4 NFB isolates (B40, NB16, F4969, and F5603) (Harrison et al., 2005; Sarker et al., 2000). The stock culture of each isolate had been maintained in cooked meat medium (Difco, Becton Dickinson, Spark, MD) at −20 °C. Bacterial growth was revived by inoculating 0.1 ml cooked meat culture into 10 ml fluid thioglycollate (FTG) medium (Difco) and incubating overnight (18 h) at 37 °C.

2.2. Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared as described previously (Paredes-Sabja et al., 2008). Briefly, 0.4-ml aliquots of an actively growing FTG cultures were inoculated into 10-ml freshly prepared Duncan Strong (DS) sporulation medium (1.5% protease peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic [Na_2HPO_4; anhydrous], 0.4% soluble starch) (Duncan and Strong, 1968) and incubated for 24 h at 37 °C. A large number of *C. perfringens* spores was prepared by scaling up the aforementioned procedure and spores were purified by repeated washing with cold sterile distilled water until the spore suspensions were >99% free of sporulating cells, cell debris, and germinating spores as observed under a phase contrast microscope. Free spores were suspended in sterile distilled water to obtain a final optical density at 600 nm (OD_{600}) of ~6 and stored at −20 °C until used (Paredes-Sabja et al., 2008).

2.3. Spore germination

The germinants used in this study consisted of potassium chloride (KCl; Fisher Scientific, Fair Lawn, NJ), L-asparagine (Sigma–Aldrich, Co., St. Louis, MO), L-glutamine (Sigma), and L-cysteine hydrochloride, monohydrate (J.T. Baker, Mallinckrodt Baker, Inc. Philipsburg, NJ) that was prepared in single or in various combinations of ingredients. All germinant solutions were prepared with 25 mM Na_2HPO_4 buffer adjusted to pH 7.0 and autoclaved (121 °C, 20 min). Germination assay was performed as previously described with some modifications. Briefly, spore suspensions were heat-activated at 80 °C, 10 min for FP isolates or 75 °C, 10 min for NFB isolates (Paredes-Sabja et al., 2008). Then, 0.1 ml of heat-activated spores (to the final concentration of OD_{600} ~1.0) were cooled in water bath at ambient temperature for 5 min before mixing with 0.5 ml of the pre-warmed germinant equilibrated at 40 °C or at room temperature (RT) (20 ± 2 °C). Spore germination was routinely monitored by measuring the decrease in OD_{600} (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA) in every 10 min intervals, as spores loss their refractility upon germination. Spore germination was also confirmed by phase contrast microscopy, as germinating spores become phase dark, whereas dormant spores remain phase bright under microscope. Results were expressed as percentage decrease of OD_{600} relative to the initial values (Paredes-Sabja et al., 2008).

2.4. Inactivation of spores in suspension

In all inactivation assays, 0.1 ml of *C. perfringens* spores (~10^8 CFU/ml) was induced to germinate with 0.5 ml of selected pre-warmed germinant or 25 mM Na_2HPO_4 buffer (pH 7.0) (Control) as described in Section 2.3. Spore germination was performed at 40 °C or at RT for 30 min before mixing with disinfectants. All disinfectants were prepared with sterile distilled water to the desired concentrations and used within 30 min after preparation. Inactivation experiments of the dormant (germinated with Na_2HPO_4 buffer) and germinating (germinated with AK) spores were performed in parallel for every disinfectant at ambient temperature. In all experiments, at least 100-fold dilution of the reaction mixtures were made immediately in 25 mM Na_2HPO_4 buffer (pH 7.5) in order to terminate the action of tested disinfectants. Preliminary results indicated that this neutralization method was effective and resulted in no residual inhibitory effect in the recovery medium (data not shown). To verify the germination abilities of different spore batches, 0.1 ml spore suspensions were heat-activated and germinated with 0.5 ml of buffer or germinant solutions for 30 min at both tested temperature and decrease in OD_{600} measured. There was no difference in the germination of spores prepared in different batches.

Ethanol is generally used for disinfecting the high-risk areas in food processing to limit water usage in order to prevent bacterial growth and spread of pathogens (Holah, 2003). The effect of initiation of germination on sensitivity to 70% (v/v) ethanol was assessed by inoculating 0.15 ml of germinated spores into 0.35 ml of absolute ethanol (100%) to give the final concentration of 70% (v/v) ethanol. After 5 min exposure at RT, 0.1 ml samples were serially diluted in 25 mM Na_2HPO_4 buffer (pH 7.5) and plated onto BHI agar to enumerate the number of viable cells.

Iodophore, the iodine-based sanitizer, have been reported to have a broad sporicidal activity against various spore-forming bacteria (Cords et al., 2005). In this study, the sporicidal activity of iodophore sanitizer against spores of *C. perfringens* isolates had been evaluated at 2 concentrations recommended by the manufacturer. Iodophore solution was prepared by diluting the appropriate amount of commercial iodophore sanitizer containing 1.6% titratable iodine (BTF® iodophore sanitizer; National Chemicals, Inc., Winona, MN) with sterile distilled water. The 0.1 ml of germinated spores were mixed with 0.5 ml of 30 ppm (mg/L) or 15 ppm iodophore to give a final concentrations of 25 ppm or 12.5 ppm titratable iodine, respectively. Spores were exposed to sanitizer for 2, 5, and 10 min and 0.1 ml were serially diluted in phosphate buffer and plated for microbial analysis.

Quaternary Ammonium Compounds (Quats) has been broadly used to disinfect food contact surfaces and food processing equipment due to its non-corrosive, environmental and user-friendly characteristics (Holah, 2003). Quats (SANI - 512 containing 10% (v/v) active ingredient, CANI Inc., Lansdale, PA) was prepared by diluting appropriate amount of Quats yielding the concentration of 240 ppm. The 0.1 ml of germinated spores were mixed with 0.5 ml of Quats solution to give a final concentration of 200 ppm. After

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