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Variability in resistance to Cold Atmospheric Plasma (CAP) and Ultraviolet light (UV) and multiple stress resistance analysis of pathogenic verocytotoxigenic *Escherichia coli* (VTEC)

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ABSTRACT

This study assessed the resistance to Ultraviolet light (UV) and Cold Atmospheric Plasma (CAP) of ten verocytotoxigenic *Escherichia coli* (VTEC) isolates and two laboratory non-pathogenic *E. coli* strains and monitored the effects that UV and CAP have on VTEC molecular composition by using Fourier transform infrared (FTIR) spectroscopy. In addition, serotype-related resistance patterns to different stresses (acid, alkaline, heat, high hydrostatic pressure, UV, CAP and biofilm formation ability) were determined through multivariate analysis of stress resistance parameters. While UV treatments were very effective in inactivating VTEC, with >5 log reductions at the lowest intensity tested (15 mJ) and low intraspecies variability, the efficacy of CAP treatments was more limited, with in general <3 log reductions after a treatment of up to 3 min in the lab-scale CAP equipment and larger interstrain variability. Two VTEC strains with non-sense mutations in the global stress response regulator *rpoS* showed an increased sensitivity to CAP at short treatment times, what suggests that RpoS is important for the cellular defensive response to this technology of preservation. Stepwise Discriminant Analysis (SDA) of FTIR spectra allowed the differentiation of untreated and treated samples based on a subrange of the spectral region w_4 (1200 to 900 cm^{-1}), which suggests that the damage inflicted to the cells is focused, both for UV and CAP treatments, in nucleic acids and the cell wall. Principal Component Analysis (PCA) of stress resistance parameters allowed the identification of significant associations between VTEC serotypes and their stress resistance patterns. Strains of the serotype O157, more commonly associated with human disease, were in general more resistant to food-related stresses than strains of other serotypes when they had a functional RpoS, emphasizing the influence that stressors may have in the epidemiology, ecology and disease-causing potential of VTEC.

1. Introduction

Verocytotoxigenic *Escherichia coli* (VTEC), characterized by the production of Shiga toxins, are a common cause of food-borne illness episodes that can lead to severe health complications and even death. In the European Union, 6043 confirmed cases of VTEC infection were reported in 2013, with a notification rate of 1.59 cases per 100,000 population. This rate was 5.9% higher than that in 2012. The main food vehicles implicated as source of infection were bovine meat, meat products, vegetables, juices and cheese (EFSA, 2015).

Over 150 different VTEC serotypes have been implicated in cases of infection. While the majority of reported cases are normally linked to serotype O157, other serotypes (e.g. O45, O26, O91, O103, O104, O111, O121 and O145) are emerging as zoonotic agents (Bielaszewska et al., 2011; L'Abee-Lund et al., 2012; Manning et al., 2007; Moretro et al., 2010; Prager, Annemuller, & Tschape, 2005; Scheutz et al., 2011). In the European Union, the most commonly reported VTEC serotype in 2013 was VTEC O157 (48.9% of cases of infection), followed by serotypes O26 (12.8%), O103 (4.3%), O145 (2.6%), O91 (2.5%), O111 (2.1%), O146 (2.0%), O128 (1.1%) and others (<1.0%) (EFSA, 2015). In the USA, the Centers for Disease Control and Prevention (CDC) has designated serotypes O26, O45, O103, O111, O121 and O145 as the most common non-O157 VTEC serotypes, accounting for more than 70% of VTEC infections from 1983 to 2002 (Brooks et al., 2005).

Intraspecies variability in VTEC resistance to food-related stresses and food preservation technologies has been recently characterized, for instance for acid pH (Uhlich, Sinclair, Warren, Chmielecki, & Fratamico, 2008; Waterman & Small, 1996), alkaline pH (Bhagwat et al., 2006), heat (Benito, Ventoura, Casadei, Robinson, & Mackey, 1999), high hydrostatic pressure (HHP) (Robey et al., 2001) and pulsed electric fields (Somolinos, Garcia, Mañas, Condón, & Pagan, 2008), in an attempt to evaluate whether the variations in transmission and/or virulence potential among *E. coli* serotypes can be attributed, at least in part, to variations in their resistance to adverse stress conditions prevailing throughout the food chain. Heterogeneity in stress resistance might

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affect the potential transmission to humans of VTEC by selecting well-adapted strains belonging to clinically-relevant genotypes. Most studies have predominantly assessed the resistance variability within the non-sorbitol fermenting serotype O157:H7, while little is known about the variation among other serotypes. A recent research article by our research team has evaluated the resistance of a collection of field VTEC isolates (O157 and non-O157, including O26, O103, O145 and O111 strains) to acid, alkaline, heat and HHP treatments, showing that variability in resistance was predominantly strain-dependent rather than serotype-dependent, although for all the stressful agents assayed the most resistant strain belonged to serotype O157 (Alvarez-Ordóñez et al., 2013). This study also evidenced that variability in VTEC stress resistance can be explained, at least in part, by heterogeneity in the *rpoS* status. Indeed, two strains that showed non-sense mutations in this stress regulator were sensitive to multiple stresses.

VTEC intraspecies variability in resistance to emerging non-thermal inactivation technologies used for decontamination of food or surfaces, such as Ultraviolet light (UV) and Cold Atmospheric Plasmas (CAP), has not been investigated yet. The importance of the alternative sigma factor RpoS for VTEC resistance towards such novel technologies is not known either.

This article is aimed at (i) assessing the resistance against UV and CAP of ten field VTEC isolates and two non-pathogenic *E. coli* strains previously characterized in Alvarez-Ordóñez et al. (2013); (ii) monitoring the effects of UV and CAP treatments on VTEC molecular composition by using Fourier transform infrared spectroscopy (FTIR); and (iii) determining the relationships between different stress resistance patterns and serotypes using multivariate analysis and integrating in the analysis the experimental results of our previous study (Alvarez-Ordóñez et al., 2013).

2. Materials and methods

2.1. Bacterial strains and culture conditions

Ten VTEC strains (clinical strains associated with outbreaks or individual clinical cases) and two non-pathogenic *E. coli* strains were used throughout the study (Table 1). Cultures were maintained in cryovials at −80 °C. Bacteria were resuscitated in tubes containing 10 ml of Brain Heart Infusion (BHI) (Oxoid) by incubation at 37 °C for 24 h followed by streaking on BHI agar (BHIA) plates, which were incubated under the same conditions. Stationary-phase bacterial suspensions were prepared by inoculating 10 ml of fresh BHI with an isolated colony and incubating it for 16 h at 37 °C, which resulted in stationary phase cultures with approximately 10^9 cells/ml, which were then used for UV and CAP inactivation experiments.

2.2. Assessment of bacterial resistance to UV

Cell cultures were harvested by centrifugation (centrifuge 5810R Eppendorf, Eppendorf, Hamburg, Germany) for 5 min at 4000 rpm, and cellular pellets were suspended in Phosphate Buffer Saline (PBS, Sigma-Aldrich). This washing step was repeated twice. Then, 50 μl of the cell suspensions were plated on the surface of BHIA plates, and the inoculum spread over the whole surface using a sterile loop. UV treatment of inoculated plates was performed in a commercially available UV cabinet (Ultraviolet Crosslinkers, UVP, Canada) at different energy intensities (15, 60, 120 & 200 mJ). Control (UV untreated) inoculated plates, were included in all experimental trials.

Survival was monitored after incubation at 37 °C for 48 h by counting surviving colonies on BHIA plates. All experiments were performed in triplicate using three independent bacterial cultures. Statistical differences in survival among bacterial strains were evaluated by one-way ANOVA analysis combined with Tukey's range test.

Table 1
Escherichia coli strains used throughout the study.

Strain no.	Strain Id.	Source	Serotype
1	EDL933	Minced meat	O157:H7
2	E218/02	Dry-fermented sausage	O157:H7
3	MF2411	Semidry-fermented sausage (mettwurst)	O111:H–
4	MF2494	Human clinical	O103:H25
5	MF2486	Human clinical	O26
6	MF2493	Human clinical	O145
7	MF2522	Dry-fermented sausage ("Morr")	O103:H25
8	MF2499	Human clinical	O103:H–
9	C-600	–	Unknown
10	M23	–	Unknown
11	MF3582	Human clinical	O157:H–
12	MF3578	Human clinical	O103:H25

2.3. Assessment of bacterial resistance to CAP

CAP treatments were performed in a commercially available air plasma jet (CP121 Plasma Demonstrator, OMVE BV, The Netherlands) as described elsewhere (Fernandez, Shearer, Wilson, & Thompson, 2012). The CAP system used is based on a copper wire electrode configured as a large bandwidth, high impedance voltage probe. Its electrical potential is perturbed by the afterglow of a jet of excited state air produced by a high voltage discharge typically sampled at a rate of 1 kHz. The electrode is located 25 mm above the gas outlet. A grid near the electrode is held at the same potential, to remove space charge. The system was operated at atmospheric pressure, and the temperature of the samples never exceeded 35 °C. Experiments were performed using air at a flow rate of 10 l/min with the power setting 3 (approx. 1 W output power). Information on plasma diagnostics for an equivalent CAP treatment unit can be found in Mastwijk, Wichers, van Dijk, and Schuten (2009) and Mols, Mastwijk, Nierop Groot, and Abee (2013). Whatman polycarbonate membrane filters of 0.2 μpore retention, 25 mm diameter (Fisher Scientific, Loughborough, UK) placed on BHIA plates were inoculated with 30 μl of bacterial cultures (previously adjusted to approximately 10^7 CFU/ml). The inoculum was spread out on the entire upper surface of filters using a sterile loop. The filters were then allowed to dry for 15 min in a laminar flow cabinet before CAP treatment. Afterwards, inoculated membrane filters were exposed to plasma conditions at predetermined times, for a period of up to 3 min. Following CAP treatment, cells were recovered from membrane filters by transferring them to sterile universal bottles containing 10 ml of peptone water and vortexing for 1 min. Series of decimal dilutions were prepared with peptone water and aliquots of 0.1–1 ml of appropriate dilutions were plated on BHIA plates in order to calculate the number of viable cells. The number of CFU was counted after incubation on BHIA at 37 °C for 48 h (longer incubation times did not have any influence on the counts). All experiments were performed in triplicate using three independent bacterial cultures. Statistical differences in survival among bacterial strains were evaluated by one-way ANOVA analysis combined with Tukey's range test.

2.4. FT-IR spectroscopy analysis

For FTIR spectroscopy analysis cultures were grown aerobically on BHIA at 37 °C for 24 h. Cells were removed with a sterile platinum loop from the culture plate and suspended in 100 μl distilled sterile water, placed (50 μl) on a ZnSe window, and stove-dried (15 min, 50 °C). Once the film was dried, the ZnSe window was CAP or UV treated as indicated above. Infrared spectra were obtained using a FTIR Spectroscope (Perkin-Elmer System 2000 FTIR, Waltham, Massachusetts, USA). Measurements were recorded in the range 3000–700 cm^{-1}, with an interval of 1 cm^{-1}. The final spectrum was achieved averaging 25 scans.

A software application developed for the Perkin Elmer environment was used for transformation of FTIR spectra, including normalization, smoothing and first derivative (Savitzky–Golay algorithm).