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Salmonella typhimurium resistance on pulsed electric fields associated with membrane fluidity and gene regulation

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ABSTRACT

Effects of different growth temperatures on cytoplasmic membrane fluidity and phospholipids phase transition temperature (T_m) of *Salmonella* typhimurium and resistance to pulsed electric field (PEF) inactivation, as well as the expression of stress-related genes and fatty acid biosynthesis-associated genes were investigated. Results indicated that the PEF resistance of *S.* typhimurium increased as growth temperature increased. *S.* typhimurium cultivated at 10 °C exhibited the lowest PEF resistance with the reduction of 4.23 $\log_{10}$ CFU/mL, while the reduction of 2.10 $\log_{10}$ CFU/mL was found in *S.* typhimurium cultivated at 45 °C under the same PEF treatment, due to the up-regulation of the expression of *fabA* gene, which was characterized by the lowest T_m of membrane phospholipids and the greatest membrane fluidity. Although the expression of alternative sigma factors were altered by growth temperature, these genes were not essential for *S.* typhimurium to develop PEF resistance, suggesting that the PEF resistance modified by growth temperature could be caused by alterations in membrane fluidity.
Industrial relevance: Pulsed electric fields (PEF) treatment has been widely applied in nonthermal pasteurization and increasingly focused on synergistic combinations with other techniques such as thermal treatment, sonication and antibacterial agents to improve the efficacy of PEF to inactivate micro-organisms. Our results indicated that *S.* typhimurium cultivated at relatively lower temperature was easily inactivated by PEF, due to the up-regulation of the expression of *fabA* gene, which was characterized by the lowest phase transition temperature of cytoplasmic membrane phospholipids and the greatest membrane fluidity. Therefore, the underlying mechanism of alterations in PEF resistance of *S.* typhimurium induced by growth temperature was explored to achieve better understanding of microbial inactivation by PEF.

1. Introduction

Pulsed electric fields (PEF) treatment has been widely applied in areas such as food security and bioengineering since PEF has the capability of rapidly inactivating micro-organisms as well as modifying properties of biomacromolecules (Aadil et al., 2015a; Hong, Chen, Zeng, & Han, 2016a; Pakhomova et al., 2012; Zhang et al., 2015). Currently, with respect to the application of food pasteurization, PEF as a non-thermal processing technique has been increasingly focused on the synergetic pasteurization with other techniques such as thermal treatment, sonication and antibacterial agents (Aadil et al., 2015b; Saldana, Monfort, Condon, Raso, & Alvarez, 2012).

The cytoplasmic membrane is considered as the primary target for PEF treatment (Aronsson, Ronner, & Borch, 2005; Chueca, Pagan, & Garcia-Gonzalo, 2015; Wouters, Bos, & Ueckert, 2001). Electroporation induced by PEF treatment is recognized as the main event in bacterial inactivation (Coster & Simmermann, 1975). Studies on electropermeability illustrated that pores in membrane could be reversible or irreversible (Saulis, 2010). Reversible pores could be induced by mild electric field strength and result in sublethal injuries, while irreversible pores could be generated by high electric field intensity and lead to cell death (Garcia, Gomez, Manas, Raso, & Pagan, 2007; Saldana, Alvarez, Condon, & Raso, 2014). Although large quantities of studies on bacterial inactivation by PEF have already been conducted (Guionet et al., 2015; Jin, Guo, & Yang, 2014; Mosqueda-Melgar, Elez-Martinez, Raybaudi-Massilia, & Martin-Belloso, 2008), the mechanism of bacterial inactivation by PEF is not fully understood.

Bacteria exhibited different PEF resistance after exposure to various external stresses, such as extreme temperature, lower pH, and hyperosmosis (Saldana et al., 2014). Previous research demonstrated that bacterial PEF resistance was greatly affected by culture temperature. Alvarez, Pagan, Raso, and Condon (2002) illustrated that *Listeria monocytogenes* cultivated at 4 °C was less likely to develop PEF resistance than those cultivated at 35 °C. In addition, the occurrence of modification in lipid composition of membrane and membrane fluidity contributed to bacterial adaptation to external stress (Davydova et al., 2016; Yang, Khoo, Zheng, Chung, & Yuk, 2014), which may result in

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change of bacterial PEF resistance. However, little is known about the relationship among the PEF resistance, growth temperature and membrane fluidity.

Bacterial resistance to external stimulation could be largely ascribed to related gene regulation (Abee & Wouters, 1999; Somolinos, Garcia, Manas, Condon, & Pagan, 2008). Alterations in related gene expression have been investigated to illustrate bacterial stress response by several studies (Somolinos, Espina, Pagán, & Garcia, 2010; White-Ziegler et al., 2008; Yang et al., 2014). These results demonstrated that the stress responses might be regulated by alternative sigma factors (*rpoS*, *rpoE* and *rpoH* genes), which played an important role in protecting cells from environmental stress and repairing damage. More information is required on the correlation between alternative sigma factors at the transcriptional level and the PEF resistance of *S.* typhimurium. In addition, since bacterial inactivation by PEF is intimately related to the generation of pores in the membrane and its phospholipids composition, we assumed that different growth temperatures might influence the expression of genes related to membrane phospholipids composition, for instance, the synthesis of unsaturated fatty acid (USA) derived from saturated fatty acid (SFA) is mainly regulated by 3-hydroxydecanoyl acyl carrier protein (ACP) dehydratase (encoded by *fabA*) (Magnuson, Jackowski, Rock, & Cronan, 1993; Zhu, Choi, Schweizer, Rock, & Zhang, 2006). Cyclopropane fatty acyl phospholipid synthase (encoded by *cfa*) is involved in the formation of cyclopropane fatty acid (CFA) (Kim et al., 2005) and malonyl CoA:ACP transacylase (encoded by *fabD*) is associated with the formation of malonyl ACP transformed by malonyl CoA (Kumari, Saxena, Tiwari, Tripathi, & Srivastava, 2013), which may contribute to the modification in PEF resistance of *S.* typhimurium.

The objective of this study was to explore the underlying mechanism of *S.* typhimurium PEF resistance modified by growth temperature based on investigating the effect of alterations in cytoplasmic membrane fluidity induced by growth temperature on the PEF resistance of *S.* typhimurium. In addition, the changes in expression of stress-related genes and fatty acid biosynthesis-associated genes were determined to identify the role of these genes in the modification in PEF resistance of *S.* typhimurium.

2. Materials and methods

2.1. Materials

Salmonella typhimurium strain (ATCC 14028) was purchased from the American Type Culture Collection (Manassas, VA, USA)0.1, 6-Diphenyl-1, 3, 5-hexatriene (DPH) was purchased from Aladdin Industrial Corporation (Shanghai, China). All other chemicals including tryptic soy broth, yeast extract, peptone and agar, magnesium chloride, hydrochloric acid, potassium chloride were obtained in Guangzhou, China.

2.2. Culture condition and modeling of growth curves

A broth subculture was prepared by inoculating a flask containing 100 mL of sterile tryptic soy broth 0.6% (*w*/*v*) of yeast extract (TSBYE), followed by incubation at 37 °C for 10 min at orbital shaker (200 rpm; OS-200, Hangzhou Allsheng Instruments Co. Ltd., Hangzhou, China). Then, 4 mL of broth subculture was transferred into sterile test tubes with 1 mL, 20% glycerol and then stored at −80 °C. Cultures in the test tube were transferred into a sterile flask containing 200 mL of TSBYE at the temperatures of 10, 20, 30, 37 and 45 °C, respectively.

The OD_{600} of cultures were recorded by a spectrophotometer (UV-1800, SHIMADZU Co. Ltd. Kyoto, Japan) at appropriate intervals after inoculation and 1 mL of cultures were serially diluted in 0.1% (*w*/*v*) sterile peptone solution. Then 0.1 mL of diluted samples were plated on tryptic soy agar plus 0.6% yeast extract (TSAYE) and incubated at 37 °C for 48 h in a controlled temperature chamber to determine the corresponding concentration of *S.* typhimurium. MATLAB software (MATLAB R2013a, The MathWorks Inc., Natick, MA) was used for the growth curves fitting by modified Gompertz equation (Gibson, Bratchell, & Roberts, 1988):

$$L(t) = A + C \times \exp[-\exp(-B \times (t - M))] \quad (1)$$

where t and L(t) denotes the growth time (h) and the log count of the number of *S.* typhimurium at time t, respectively. A denotes log count as t decreases indefinitely (log CFU/mL), C denotes the log amount of growth as t increases indefinitely (log CFU/mL), M denotes the time when the absolute growth rate reaches maximum (h), and B denotes the relative growth rate at M. The growth parameters was computed according to the fitted parameters, such as final population density (FPD = A + C), generation time (GT = log (2e/BC)), growth rate (GR = BC/e) and lag time (LT = (M − 1)/B).

2.3. PEF treatment

Prior to PEF treatment, a table-top low speed refrigerated centrifuge (4000 g, 5 min, 4 °C) (JW-3021HR, Anhui Jiaven Equipment Industry Co. Ltd., Hefei, China) was used to obtain *S.* typhimurium cells after reaching stationary phase (OD_{600} = 1.5). The supernatants were removed and the pellets were re-suspended in sterile deionized water adjusted to a concentration of 10^9 CFU/mL of *S.* typhimurium. The pH value of samples was maintained at 6.9 ± 0.1. In order to avoid the effect of electrical conductivity and thermal treatment, sterile potassium chloride solution (2 mol/L) was used to adjust the electrical conductivity to 180 ± 1 μS/cm.

For PEF processing, the initial temperatures (10 °C, 25 °C, 40 °C) of samples were controlled by a heat exchanger (DLSK-3/10, Ketai Instrument Co. Ltd., Zhenzhou, China). Then the samples were pumped through a rotary pump (323E/D, Watson-MarlowInc., MA, USA) into a continuous-flow PEF system described in previous studies (Hong, Zeng, Buckow, Han, & Wang, 2016b; Zhao, Zeng, Sun, & Liu, 2013). The PEF equipment and processing parameters were as follows: bipolar square wave was used; pulse frequency was 1 kHz; pulse width was 40 μs; PEF chamber was consisted of two parallel titanium plate electrodes with electrode gap of 0.30 cm and flow volume of 0.02 mL; the flow rate was set at 40 mL/min; the electric field strength was 25 kV/cm and the times of treatment cycle was 1. The PEF treatment time t (s) was computed with the following equations:

$$t = n \times N_p \times W_p \quad (2)$$

$$N_p = \frac{V \times f}{F} \quad (3)$$

where n represents the times of treatment cycle, N_p represents the number of pulses obtained in the chamber, W_p represents the pulse duration (μs), f represents the pulse frequency (Hz), V represents the volume of the chamber (mL) and F represents the flow rate (mL/s).

In the current experiment, the treatment time was 1.2 ms based on Eqs. (2) and (3). The starting and final temperatures were recorded by a thermocouple thermometer (WRE/E, Xingtai Instrument Co. Ltd., Jiangsu, China). Control sample was obtained with similar operation without any treatment.

2.4. Detection of inactivation

Suspensions of PEF-treated and control cells were serially diluted, plated and incubated as mentioned above. The inactivation of *S.* typhimurium was evaluated by calculating the $\log_{10}$ reduction in the treated samples compared to the control sample.

2.5. Determination of membrane fluidity

Membrane fluidity was detected using DPH as the fluorescent probe by determining fluorescence anisotropy as previously described (Zhang,