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Effect of temperature on pathogenic and non-pathogenic *Vibrio parahaemolyticus* biofilm formation

Xueying Song [a, 1], Yuejiao Ma [a, 1], Jiaojiao Fu [a], Aijing Zhao [a], Zhuoran Guo [a], Pradeep K. Malakar [a], Yingjie Pan [a, b, c], Yong Zhao [a, b, c, *]

[a] College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China
[b] Shanghai Engineering Research Center of Aquatic-Product Processing & Preservation, Shanghai 201306, China
[c] Laboratory of Quality & Safety Risk Assessment for Aquatic Product on Storage and Preservation (Shanghai), Ministry of Agriculture, Shanghai 201306, China

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ABSTRACT

Biofilm formation is crucial for the environmental survival and transmission of *Vibrio parahaemolyticus*, an important food-borne pathogen in seafood. The biofilm developmental process of pathogenic (n = 22) and non-pathogenic (n = 17) *V. parahaemolyticus* strains on polystyrene microtiter plates under 15 °C, 25 °C and 37 °C was investigated using crystal violet staining, and validated by confocal laser scanning microscopy. The results indicated that biofilm developmental process at 15 °C and 25 °C were divergent, biofilm formation increased continuously at 15 °C, while at 25 °C biofilm formation increased gradually and peaked at 12 h. Also the biofilm formation was dramatically elevated at 25 °C in comparison with that at 15 °C and 37 °C. Additionally, pathogenic strains, on average, formed more biofilm than non-pathogenic strains at all temperatures measured. Moreover, extensive strain variability was observed during biofilm formation and indexed using the coefficient of variation (CV). This index increased with increasing temperature and this index, at all temperatures, peaked after 12 h. The results of this study provide insight into the developmental process of biofilm, which allow us to further optimize strategies to control *V. parahaemolyticus* biofilm in food industry.

1. Introduction

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a Gram-negative, halophilic food-borne pathogen, known as a leading cause of seafood-derived food poisoning (Broberg, Calder, & Orth, 2011; Yeung & Boor, 2004). *V. parahaemolyticus* is generally isolated from a variety of seafoods including fish, shrimp, oyster, scallop and crab. Consumption of raw or undercooked seafood contaminated with *V. parahaemolyticus* may lead to development of acute gastroenteritis (Su & Liu, 2007; Xu, Wang, Sun, Liu, & Li, 2013). It has been reported that in 802 outbreaks of food-borne disease in the eastern coast of China, 40.1% were related to *V. parahaemolyticus* (Chao et al., 2010).

In most natural or food-processing environments, *V. parahaemolyticus* survive by forming biofilm (Costerton, Stewart, & Greenberg, 1999). Bacterial biofilms are sessile communities with microorganisms adhere to biotic or abiotic surface and embedded within a matrix of extracellular polymeric substances, including exopolysaccharides, proteins and extracellular DNA (Flemming & Wingender, 2010; Hall-Stoodley, Costerton, & Stoodley, 2004). Biofilm cells are more resistant to disinfectants and antibacterial agents than their planktonic form, so the ability of this pathogen to form biofilm is important for enhancing environmental survival and increasing infectivity and transmission (Elexson et al., 2014; Kadam et al., 2013).

Biofilm formation by *V. parahaemolyticus* is a complex and differentiated dynamic process involved in both cell - surface and cell - cell interactions, which determine their structure, function and composition (Karunakaran, Mukherjee, Ramalingam, & Biggs, 2011; Lianou & Koutsoumanis, 2012). Such interactions are affected by various environmental conditions such as incubation temperature, pH, a_w and contact surfaces. Furthermore, *V. parahaemolyticus* experiences seasonal and inter-annual changes

* Corresponding author. College of Food Science and Technology, Shanghai Ocean University, Shanghai 201306, China.
E-mail address: yzhao@shou.edu.cn (Y. Zhao).
[1] These authors contributed equally to this study.

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in temperature within aquatic reservoirs, and it has been demonstrated that *V. parahaemolyticus* outbreaks are highly correlated with sea surface temperature and seasonal temperature fluctuation (Duan & Su, 2005; Turner, Malayil, Guadagnoli, Cole, & Lipp, 2014). The importance of temperature effects on biofilm formation has been ascertained for many environmental microorganisms, such as *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* (Kadam et al., 2013; Lianou & Koutsoumanis, 2012; Di Ciccio et al., 2015; Stepanović, Ćirković, & Ranin, 2004; Yang et al., 2016.). Several high resolution imaging techniques (such as confocal laser scanning microscopy and scanning electron microscopy) have been developed and used to explore the characteristic of biofilm formation, however, to our knowledge, the influence of temperature on biofilm development for pathogenic and non-pathogenic *V. parahaemolyticus* has not been studied.

Listeria monocytogenes strains from both serotype 1/2b and 1/2a formed more biofilm than serotype 4b strains (Kadam et al., 2013), and additional studies have also demonstrated differences in biofilm formation between serotypes of pathogenic and non-pathogenic strains (Díez-García, Capita, & Alonso-Calleja, 2012; Nilsson, Ross, & Bowman, 2011). Besides, previous studies (Lianou & Koutsoumanis, 2012; Nilsson et al., 2011; Rivas, Dykes, & Fegan, 2007) have also discovered that biofilm formation among strains is variable. But no research about whether the strain variability exists on the developmental process of biofilm by *V. parahaemolyticus* at different temperatures, so increased knowledge about the variation during biofilm formation is needed to optimize preventative measures and minimize the risk that biofilm production by food-borne pathogens presents on food industries.

Therefore, the main objective of the present study was to explore the dynamic process (0–48 h) of biofilm formation by pathogenic (n = 22) and non-pathogenic (n = 17) *V. parahaemolyticus* strains at different temperatures (15 °C, 25 °C and 37 °C). The relationship between biofilm formation and strain pathogenicity, and the strain variability during biofilm formation were also evaluated.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

A total of 39 *V. parahaemolyticus* strains, isolated from patients (4 strains) and shrimp (35 strains) were used in this study, including 22 pathogenic strains and 17 non-pathogenic strains, the genotype and origins of each strain was listed in Table 1.

Stock cultures of the strains were stored at −80 °C in Tryptic Soy Broth (TSB, Land Bridge Technology, Beijing, China) with 50% (v/v) glycerol. Prior to each experiment in our study, the strains were activated by transferring them into Thiosulfate citrate bile salts sucrose agar (TCBS agar, Land Bridge Technology, Beijing, China) and incubating them overnight at 37 °C, then strains were activated again by transferring single colony from the TCBS agar into 9 mL of TSB containing 3% NaCl and inoculated overnight at 37 °C with shaking at 200 rpm. Subsequently, the *V. parahaemolyticus* cultures were diluted with TSB (3% NaCl) to obtain a final optical density at 600 nm (OD_{600}) of 0.4 for subsequent experiment.

2.2. Biofilm formation assay

Biofilms were prepared as described previously (Kadam et al., 2013) with minor modifications. Statistic biofilms were grown in 24 - well polystyrene microtiter plates (Sangon Biotech Co., Ltd., Shanghai, China) and the plate was sealed using plastic wrap to prevent water evaporation. More specifically, the 24-well microtiter plates were filled with 990 microlit TSB (3% NaCl), followed by addition of 10 microlit aliquots of the *V. parahaemolyticus* cultures (OD_{600} = 0.4) to each well, each strain was tested in three replicate. The wells containing uninoculated TSB served as negative control. To study the dynamic process of biofilm formation, the microtiter plates were incubated statically at different temperatures (15 °C, 25 °C and 37 °C) for various time (2 h, 8 h, 12 h, 24 h and 48 h) without shaking. After incubation, biofilm production was quantified using crystal violet staining method as described previously by Stepanović, Vuković, Dakić, Savić, and Švabić-Vlahović (2000) with some modification. Specifically, the suspension of the plates' wells was discarded and the wells were gently washed three times with 1 mL of 0.1 M phosphate buffer (PBS) to remove non-adherent cells, then the microtiter plates were air - dried for 10 min and stained with 1 mL of 0.1% (w/v) crystal violet (Sangon Biotech Co., Ltd., Shanghai, China) for 30 min at room temperature, and then washed three times with 1 mL of 0.1 M PBS to remove excess crystal violet. After drying for 30 min at room temperature, crystal violet stained biofilms was solubilized using 2 mL of 95% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 30 min. The optical density of each well was measured at wavelength of 570 nm using the Bio Tek Elisa. OD values of the polystyrene microtiter plates cultured with fresh medium (TSB, 3% NaCl) only were used as negative controls, and the difference between the optical density of tested strains (OD_{570nm}) and negative control samples (ODc) were used to characterize the biofilm-forming ability of the tested strains. The capacity of biofilm formation of strains was classified into four categories according to Stepanović et al. (2000), and the specific criterion was as follows: non-biofilm producer ($OD_{570nm} \leq$ ODc), weak biofilm producer (ODc < $OD_{570nm} \leq$ 2-times ODc), moderate biofilm producer (2-times ODc < $OD_{570nm} \leq$ 4-times ODc), and strong biofilm producer (OD_{570nm} > 4-times ODc).

Table 1
The genotype and origins of 39 *V. parahaemolyticus* strains used in this study.

Pathogenic strains			Non-pathogenic strains		
Strains	Genotype	Origin	Strains	Genotype	Origin
VP-S1	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S8	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S2	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S13	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-C3	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	IMCAS	VP-S14	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S4	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S15	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-C5	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	IMCAS	VP-S16	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S6	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S17	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-C7	tdh$^-$/trh$^+$/tlh$^+$	IMCAS	VP-S18	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S9	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S19	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S10	tdh$^-$/trh$^+$/tlh$^+$	Shrimp	VP-S20	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S11	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S21	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S12	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S25	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S22	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S33	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S23	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S34	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S24	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S35	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S26	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S36	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S27	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S37	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S28	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp	VP-S38	*tdh*$^-$/*trh*$^-$/*tlh*$^+$	Shrimp
VP-S29	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp			
VP-S30	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp			
VP-S31	*tdh*$^+$/*trh*$^-$/*tlh*$^+$	Shrimp			
VP-S32	tdh$^+$/trh$^-$/tlh$^+$	Shrimp			
VP-C39	tdh$^+$/trh$^+$/tlh$^+$	CDC			

CDC, Centers for Disease Control and Prevention. IMCAS, Institute of Microbiology Chinese Academy of Sciences.

2.3. Confocal laser scanning microscopy (CLSM) imaging

A typical strain of VP-C7, which exhibited strong biofilm-forming capacity at various temperatures, was chosen for further visualization of biofilm architectures using confocal laser scanning microscopy (CLSM). Static biofilms were prepared as described