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Biofilm formation by *Vibrio parahaemolyticus* on food and food contact surfaces increases with rise in temperature

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A B S T R A C T

Vibrio parahaemolyticus is recognized as a human foodborne pathogen that is mostly associated with seafood. This pathogen can form a mature biofilm on food and food contact surfaces during food processing. The present study investigated *V. parahaemolyticus* biofilm formation at various temperatures on shrimp, crab, and stainless steel coupons, using the biofilm formation index (BFI) method on microtiter plates. The results were also confirmed by field emission scanning electron microscopy. Both the BFI values and cultural counts revealed that *V. parahaemolyticus* biofilm formation was stronger at higher temperatures than at lower temperatures. Biofilm formation differed according to the growth surface type and growth temperature. It was found that higher temperatures (15–37 °C) induced stronger biofilm formation whereas 4 and 10 °C resulted in attachment of the bacterial cells as monolayers. It could be concluded that temperatures of 25–37 °C result in significantly stronger biofilm formation as well as exoprotease and AI-2 production on food and food contact surfaces, indicating that these temperatures might be threatening conditions for food safety.

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1. Introduction

The gram-negative bacterium *Vibrio parahaemolyticus* is associated with seafood-borne illness and is recognized as a human pathogen. It is isolated from a variety of raw seafoods, particularly shellfish. The pathogen is a common cause of foodborne illnesses in many Asian countries, including China, Japan, and Korea (Su & Liu, 2007). Food-poisoning outbreaks attributed to *V. parahaemolyticus* occur at a high frequency in Asia. In Korea, according to a recent report, the bacterium was involved in 9–16% of the total food-poisoning cases reported. In particular, on a national scale, *V. parahaemolyticus* food-poisoning outbreaks occurred in 78 patients in Korea (MFDS, 2014). This bacterial species usually attaches to underwater surfaces and is generally isolated from a variety of raw seafoods. It is mainly associated with outbreaks related to the consumption of shellfish (Xu, Wang, Sun, Liu, & Li, 2013). Recently, *V. parahaemolyticus* has been implicated in an outbreak due to the consumption of raw oysters (Newton et al., 2014).

Biofilms are architecturally complex assemblies of microorganisms on or in biotic or abiotic surfaces and interfaces, characterized by interactions between the populations. Biofilms contain exopolymeric substances and survive as self-organized, three-dimensional structures that exhibit altered phenotypic and genotypic characters (Jahid & Ha, 2012; Mizan, Jahid, & Ha, 2015). *V. parahaemolyticus* is known to form biofilms on seafood (Rajkowski, Fratamico, Annous, & Gunther, 2009). Cell attachment and biofilm formation have also been studied in some of the marine vibrios, including *Vibrio harveyi* (Karunasagar, Otta, & Karunasagar, 1996), *Vibrio cholerae* (Faruque et al., 2006), *Vibrio vulnificus* (Joseph & Wright, 2004), and *V. parahaemolyticus* (Elexson et al., 2013). Like other biofilm-producing microorganisms, *V. parahaemolyticus* is capable of producing distinct types of adherence factors that enable the bacterium to adhere to the surface and initiate biofilm formation (Donlan, 2002).

The importance of different temperature effects on virulence factors and biofilm formation has been ascertained for different microorganisms, such as *Enterococcus* spp. (Jahan & Holley, 2014), *Salmonella* spp. (Stepanovic, Cirkovic, Ranin, & Svabić-Vlahović, 2004), *V. vulnificus* (McDougald, Lin, Rice, & Kjelleberg, 2006), and *Listeria monocytogenes* (Di Bonaventura et al., 2008). As these are environmental microorganisms, it would be very common for their survival and biofilm formation to be modulated by temperature.

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Quorum sensing is the population-density-dependent manner by which microorganisms communicate and coordinate with intraspecies and interspecies members by secreting signaling molecules. The importance of quorum sensing for foods has been reviewed by several authors (Bai & Rai, 2011; Mizan et al., 2015; Skandamis & Nychas, 2012; Smith, Fratamico, & Novak, 2004). When the bacterial population reaches a specific concentration, they secrete autoinducers (AIs) into the surrounding environment. Once a specific concentration of AIs is reached, the molecules diffuse back into their producing bacteria and induce target genes, essentially at the stationary phase, which consequently change the behavior of the bacteria (Daniels, Vanderleyden, & Michiels, 2004). The objective of this study was to assess the biofilm formation, exoprotease and AI-2 production of *V. parahaemolyticus* on stainless steel (SS) and seafood (crab and shrimp) surfaces at different temperatures.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and growth conditions

Vibrio parahaemolyticus KCTC 2471 (isolated from a case of food poisoning), KCTC 2729 (isolated from a patient suffering from “Shirasu” (the fry of sardine boiled and sold in a half dried state)” food poisoning, and ATCC 33844 (isolated from a patient with food poisoning) were used as a cocktail in this study. Prior to each experiment in our laboratory, the strains were activated by transferring them from stocks stored at −80 °C to thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and incubating them overnight at 30 °C. A single colony from each plate was inoculated into 5 mL of tryptic soy broth (TSB; Difco) containing 2.5% NaCl and incubated overnight at 30 °C in a shaking incubator (VS-8480; Vision Scientific, Daejeon Si, South Korea) at 220 rpm. Subsequently, the *V. parahaemolyticus* cultures were centrifuged at 11,000*g* for 10 min, washed, and resuspended in fresh LB broth (2% NaCl; Difco Laboratories) to obtain a final optical density at 600 nm (OD_{600}) of 1.0. These cultures were diluted as required and used in subsequent planktonic growth, biofilm formation, exoprotease, and quorum sensing assays at different temperatures of 4, 10, 15, 20, 25, 30, and 37 °C. These cultures are referred to as “standardized cultures” throughout the text.

2.2. Quantitative biofilm formation assay in microtiter plates

This experiment was conducted as described previously by Jahid, Lee, Kim, and Ha (2013) with some modification. After the cultures had been grown in 2.5% NaCl-containing TSB for 24 h with shaking, the standardized cultures were diluted (1:50) in 2.5% NaCl-containing TSB and 100-μL aliquots were placed in each well of 96-well polystyrene microtiter plates (Becton Dickinson Labware; Becton, Dickinson and Company). The microtiter plates were incubated at different temperatures (viz., 4, 10, 15, 20, 25, 30, or 37 °C) for 72 h without shaking. After incubation, the optical density of the total bacteria in the microtiter plates was measured at a wavelength of 595 nm (OD_{595}) with a microtiter plate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). The plates were washed by submersion into a small tub of water after the bacterial cultures had been discarded. The plates were air-dried overnight and stained with 125 μL of 0.1% (w/v) crystal violet dye (CV; Sigma-Aldrich, St. Louis, MO, USA) for 45 min at room temperature, and then again dried overnight. The CV was solubilized using 125 μL of 95% (v/v) ethanol at room temperature for 10 min and the absorbance was read at 570 nm using a microtiter reader. The biofilm formation index (BFI) was determined by applying the equation described by Teh, Flint, and French (2010):

$$BFI = \frac{AB - CW}{GB - GW}$$

where AB is the OD_{595} of the CV-stained attached microorganisms, CW is the OD_{595} of the stained blank wells containing microorganism-free medium only, GB is the OD_{570} of the cell growth in suspended culture, and GW is the OD_{570} of the blank well. The degree of biofilm formation was classified according to Naves et al. (2008): none ($BFI < 0.35$), weak ($0.35 \leq BFI \leq 0.69$), moderate ($0.70 \leq BFI \leq 1.09$), and strong ($BFI \geq 1.10$).

2.3. Preparation of stainless steel coupons, biofilm formation, and detachment population

Austenitic SS (Type 302; Chung-Ang Scientific Inc., Seoul, Korea) coupons (2 × 2 × 0.1 cm) were processed as described by Shen et al. (2012). *V. parahaemolyticus* cells were centrifuged, washed, and resuspended in TSB containing 2.5% NaCl. The suspension was diluted to 1:50 and inoculated into 50 mL Falcon tubes containing a SS coupon that was completely submerged in 10 mL of TSB. The tubes were incubated at 4, 10, 15, 20, 25, 30, or 37 °C for 24 h to allow biofilm formation on the SS coupons. Following the incubation, each SS coupon was transferred to a small Petri dish (55 × 12 mm) containing 1 mL of 0.1% peptone water (PW) and then agitated by rotating it clockwise and anticlockwise, using sterile tweezers. Agitation was always performed by the same person, thus it was assumed that the same amount of pressure was applied to all coupons. The removed cells were subsequently vortexed and diluted in PW for enumeration. Cell numbers were quantified after incubation on TCBS agar for 24 h.

2.4. Preparation of inoculum for food samples

The cultures in TSB containing 2.5% NaCl were centrifuged (11,000*g* for 10 min at 4 °C) and the pellets were washed with sterile phosphate-buffered saline (PBS, pH 7.2). The pellets were resuspended in the appropriate amount of PBS to make up the same final concentration of bacterial cells. These inocula were used to form biofilm on crab and shrimp coupons.

2.5. Preparation of shrimp and crab coupons, biofilm formation, and detachment population

Crab and shrimp were purchased from a local grocery store in Anseong, Korea. The shellfish were cut with a sterile laboratory scissor into 2 × 2 cm^2 coupons that were then washed with sterile distilled water to remove the flesh. Prior to inoculation with *V. parahaemolyticus*, the coupons were placed in an open sterile Petri dish and subjected to ultraviolet-C treatment for 30 min on each side to minimize the background flora. Each coupon was then submerged in 10 mL of fresh water and the bacteria were inoculated at a 1:2500 dilution. The dishes were incubated for 24 h, without shaking, at different temperatures (4, 10, 15, 20, 25, 30, 35, and 37 °C). Following incubation and for the detachment of microbial populations from the coupons, the procedures described by Jahid, Han, Srey, and Ha (2014) were applied with minor modifications. The coupons were placed in 10 mL of PW (Oxoid, UK) into a sterile stomacher bag (Whirl-Pak; Nasco, Fort Atkinson, WI, USA) and processed using a stomacher (BagMixer; Interscience, Saint-Nom-la-Bretèche, France) at the highest speed for 2 min to release the biofilm-forming bacteria from the samples. Enumeration of *V. parahaemolyticus* was carried out by serial dilution and spread plating onto TCBS agar. The plates were incubated at 37 °C for 24 h

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