International Journal of Food Microbiology 236 (2016) 123–129

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Role of extracellular matrix protein CabA in resistance of *Vibrio vulnificus* biofilms to decontamination strategies

Jin Hwan Park [1], Byungho Lee, Youmi Jo [2], Sang Ho Choi *

National Research Laboratory of Molecular Microbiology and Toxicology, Department of Agricultural Biotechnology, and Center for Food Safety and Toxicology, Seoul National University, Seoul 08826, South Korea

ARTICLE INFO

Article history:
Received 10 March 2016
Received in revised form 22 July 2016
Accepted 27 July 2016
Available online 27 July 2016

Keywords:
Vibrio vulnificus
Biofilm
CabA
Matrix protein
Decontamination strategy

ABSTRACT

Biofilms are recalcitrant and raise safety problems in the food industry. In this study, the role of CabA, an extracellular matrix protein, in the resistance of the biofilms of *Vibrio vulnificus*, a foodborne pathogen, to decontamination strategies was investigated. Biofilms of the *cabA* mutant revealed reduced resistance to detachment by vibration and disinfection by sodium hypochlorite compared to the biofilms of the parental wild type *in vitro*. The reduced resistance of the *cabA* mutant biofilms was complemented by introducing a recombinant *cabA*, indicating that the reduced resistance of the *cabA* mutant biofilms is caused by the inactivation of *cabA*. The expression of *cabA* was induced in cells bound to oyster, the primary vehicle of the pathogen. The *cabA* mutant biofilms on oyster are defective in biomass and resistance to detachment and disinfection. The bacterial cells in the wild-type biofilms are clustered by filaments which are not apparent in the *cabA* mutant biofilms. The combined results indicated that CabA contributes to the structural integrity of *V. vulnificus* biofilms possibly by forming filaments in the matrix and thus rendering the biofilms robust, suggesting that CabA could be a target to control *V. vulnificus* biofilms on oyster.

1. Introduction

Bacteria reside predominantly as sessile biofilms rather than as free-living planktonic cells in many different environments (September et al., 2007; Wimpenny et al., 2000). Biofilms of pathogenic bacteria are considered to be one of the most important causes for new outbreaks and account for 65% of bacterial infections in humans (Costerton, 2001). Biofilms are specialized and highly differentiated three-dimensional communities of bacteria encased in an extracellular polymeric matrix (EPM), a framework contributing to the organization and maintenance of biofilm structure (Karatan and Watnick, 2009). Biofilm formation provides bacteria with increased resistance to antimicrobial agents and external stress as well as to host immune defense systems during infection (Hall-Stoodley et al., 2004; Hall-Stoodley and Stoodley, 2005). It is well known that biofilms are more resistant to conventional decontamination strategies compared to their planktonic counterparts and difficult to eradicate due to their resistant phenotypes (Simões et al., 2010). Thus, biofilms are problematic in the food industry as major sources of recalcitrant contaminations, causing food spoilage and public health problems such as outbreaks of foodborne pathogens. Therefore, understanding the mechanisms involved in the formation of biofilms and maintenance of their structural integrity has become one of the most important concerns in food safety communities in order to develop efficient strategies to decontaminate biofilms in foods and food processing facilities.

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of foodborne diseases such as gastroenteritis and possibly life-threatening septicemia in individuals with underlying predisposing conditions such as liver damage, excess levels of iron, and immunocompromised conditions (Jones and Oliver, 2009; Oliver, 2015). Wound infections result from exposure to seawater or from the handling of shellfish contaminated with *V. vulnificus*. *V. vulnificus* is highly lethal as mortality from septicemia is very high (>50%) and death may occur within 1 to 2 days after the first signs of illness, and thereby is responsible for the majority of reported seafood-related deaths worldwide, (Jones and Oliver, 2009; Oliver, 2015). The primary food vehicles of the pathogen are oysters, and over 90% of infections resulting in *V. vulnificus* septicemia are associated with consumption of raw and/or undercooked oysters (Oliver, 2015). Therefore, efforts have been made to develop many postharvest processes of oysters to eliminate *V. vulnificus*: depuration (Lewis et al., 2010), high hydrostatic pressure inactivation (Kural and Chen, 2008; Ye et al., 2013), heat/cool pasteurization (Andrews et al., 2000; Melody et al., 2008), irradiation (Mahmoud, 2009), and treatment with oxidizing agents such as sodium hypochlorite (NaOCl) (Ramos et al., 2012). However, these postharvest

* Corresponding author.
E-mail address: choish@snu.ac.kr (S.H. Choi).
[1] Present address: Department of Microbiology and Environmental Toxicology, University of California - Santa Cruz, Santa Cruz, California, 95064, United States of America.
[2] Present address: Agromaterial Assessment Division, Department of Agro-Food Safety and Crop Protection, National Institute of Agricultural Science, Wanju 55365, South Korea.

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.07.032
0168-1605/

decontamination strategies are not always effective in reducing the pathogen in oysters to the levels favorable for human consumption. Although other explanations are also possible, this limited effectiveness of the standard processes developed to disinfect pathogens of planktonic state may reflect the presence of *V. vulnificus* biofilms in oysters. Consistent with this, it has been suggested that *V. vulnificus* embed themselves in oyster tissues and form biofilms to persist in oysters (Froelich and Oliver, 2013; Paranjpye et al., 2007).

It has been reported that the resistance of biofilms to hostile challenges is largely attributable to their structure determined by EPM (Flemming and Wingender, 2010). Recently, *V. vulnificus* CabA was identified and characterized as a structural protein that is distributed throughout the extracellular matrix. It was also shown experimentally that CabA is essential for biofilm formation in microtiter plates and biofilm structure in flow cells (Park et al., 2015). In this study, to further investigate the role of CabA in the development of biofilms on oysters, the primary niche of *V. vulnificus* in nature, the expression of *cabA* in the cells bound to oyster was compared with that in the planktonic cells. The structure of the wild-type and *cabA* mutant biofilms on oyster as well as *in vitro* was evaluated in terms of their resistance to decontamination strategies such as detachment by vibration (Kim et al., 2012) and disinfection by NaOCl. The structures of biofilms on the oyster shells produced by the *cabA* mutant and wild type were further examined using scanning electron microscopy (SEM). The results proposed that CabA participates in forming filaments which cluster bacterial cells together in the matrix and provide the *V. vulnificus* biofilms with resistance to the decontamination strategies, suggesting that inhibiting the synthesis and/or the activity of CabA may aid in effective reduction of *V. vulnificus* in oysters.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

The strains and plasmids used in this study are listed in Table 1. *V. vulnificus* CMCP6 and its isogenic mutant YM112, in which the *cabA* gene was deleted (Park et al., 2015), were used as the parental wild type and *cabA* mutant, respectively. For complementation of the *cabA* mutation, the plasmid pYM1109 carrying a recombinant *cabA* was transferred into the *cabA* mutant as described previously (Park et al., 2015). Unless otherwise noted, the *V. vulnificus* strains were grown in Luria-Bertani (LB) medium supplemented with 2.0% (w/v) NaCl (LBS) at 30 °C. The *Vibrio fischeri* minimal medium (Cao et al., 2012) containing glycerol (50 mM Tris-HCl, pH 7.2, 50 mM $MgSO_4$, 300 mM NaCl, 10 mM KCl, 0.33 mM K_2HPO_4, 18.5 mM NH_4Cl, 10 mM $CaCl_2$, and 32.6 mM glycerol) (VFMG) was used for biofilm formation.

2.2. Formation, detachment, and disinfection of biofilms in vitro

Biofilms on the test tube surfaces were formed and quantified using the procedure developed by O′Toole and Kolter (O'Toole and Kolter, 1998) with minor modifications. Briefly, each of 14 ml round-bottom test tubes (BD Biosciences, Erembodegem, Belgium) was inoculated with 1 ml of *V. vulnificus* cultures diluted to an A_{600} 0.05 with VFMG and then incubated for 48 h at 30 °C without shaking to form biofilms. Once the planktonic cells were gently removed, the biofilms on the tube surfaces were rinsed with phosphate-buffered saline (PBS, pH 7.4). To evaluate the resistance of the biofilms to the physical or chemical decontamination strategies, the biofilms on the tube were soaked in 1 ml PBS, and then were either vibrated using the micro mixer (Confido S-20, FINEPCR, Seoul, Korea) at the speed of 1200 rpm or treated with 10 ppm (mg/l) of NaOCl solution (Yuhan Clorox, Seoul, Korea) for various periods. The residual biofilms were rinsed with PBS, and then stained with 1.2 ml of 1% (w/v) crystal violet (CV) solution for 15 min at room temperature and quantified by elution of CV with 1.2 ml of 100% ethanol and measurement of absorbance at 570 nm (A_{570}).

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
CMCP6	Wild-type *V. vulnificus*, virulent	Laboratory collection
YM112	CMCP6 with Δ*cabA*	Park et al. (2015)
Plasmids		
pJK1113	pKS1101 with *nptI*; Ap^r Km^r	Lim et al. (2014)
pYM1109	pJK1113 with *cabA*; Ap^r Km^r	Park et al. (2015)

[a] Ap^r, ampicillin resistant; Km^r, kanamycin resistant.

2.3. Formation, detachment, and disinfection of biofilms on oyster

The fresh oysters (*Crossostrea gigas*) were purchased from a local seafood market in the winter season, scrubbed with a wire brush to remove surface dirt, and shucked with a sterile knife, after which the shells were fragmented into the size of 1 cm × 1 cm. The meat and fragmented shells were washed under running cold sterile PBS and then kept frozen in a sterile plastic bag until used. *V. vulnificus* cultures diluted to an A_{600} 0.02 with 225 ml of VFMG were incubated with 25 g of the oyster meat or shells for 24 h at 30 °C without shaking to form biofilms. To quantify the biofilm cells on the meat, the planktonic cells were removed by gently rinsing with PBS, and the meat was mixed with 100 ml of PBS and homogenized for 2 min using the Stomacher (EASY MIX, AES Chemunex, Rennes, France). The biofilm cells in the homogenate were quantified in colony forming units (CFUs) on LBS agar plates containing 100 U/ml of polymyxin B, which were selective for *V. vulnificus* cells (Cerdà-Cuéllar et al., 2000). In a similar way, after the shells were soaked in 100 ml of PBS, biofilm cells were detached from the shells by vibration at the speed of 7000 g for 2 min using the Spindle (Kim et al., 2012) and enumerated as CFUs. The Spindle vibration has been proven to effectively detach foodborne pathogens from different types of food with less destruction of the food texture (Kim et al., 2012). As a negative control, the oyster meat or shells which were incubated with 225 ml of sterile VFMG in the same conditions but without artificial inoculation of *V. vulnificus*, after which the biofilm cells were enumerated.

To evaluate the resistance of the biofilms to the physical or chemical decontamination strategies, the biofilms on the surfaces of oyster (meats or shells) were soaked in 100 ml PBS. The biofilms were vibrated using the Spindle at the speed of 7000 g for various periods and the detached biofilm cells were enumerated as CFUs as described above. Similarly, the biofilms were treated with 10 ppm (mg/l) of NaOCl solution for various periods and the residual biofilm cells were enumerated.

2.4. RNA purification and transcript analysis

V. vulnificus biofilms were formed on the surfaces of oyster as described above. Planktonic and biofilm cells, which were detached using the Spindle at the speed of 7000 g for 2 min, were harvested separately. For quantitative real-time PCR (qRT-PCR) analyses of *cabA* expression, total RNAs were isolated from the harvested cells using an RNeasy® Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from the RNAs by using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) and real-time PCR amplification of the cDNA was performed by using the Chromo 4 real-time PCR detection system (Bio-Rad Laboratories) with a pair of primers, qRTcabA_F (TTGGTTGCTGGC TCTGGTGAC) and qRTcabA_R (ACTGTCTATACGCACTGTGTCCTC) (Park et al., 2015). Relative expression levels of the *cabA* transcripts were calculated by using the 16S rRNA expression level as the internal reference for normalization.