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

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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

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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.

2.5. Scanning electron microscopy (SEM)

Biofilms on the surfaces of oyster shells were fixed, and dehydrated for SEM analysis by the procedures as previously described (Park et al., 2015). Briefly, the biofilms were fixed using a buffer [0.1 M sodium cacodylate, 0.2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, pH 7.3] for 1 h at 4 °C, washed with 0.05 M sodium cacodylate for 20 min at 4 °C, and then postfixed with 0.05 M sodium cacodylate (pH 7.3) containing 1% (v/v) osmium tetroxide for 45 min at 4 °C. Fixed biofilms were washed with distilled H_2O, dehydrated in increasing concentrations of ethanol (50, 70, 80, 90, and 100%), and then dried in hexamethyldisilazane. Dried biofilms were mounted on an aluminum stub, coated with platinum using a sputter coater (BAL-TEC SCD 005, BAL-TEC AG, Balzers, Liechtenstein), and visualized using SEM (Supra 55VP, Zeiss, Jena, Germany).

2.6. Data analyses

Averages and standard deviation (SD) were calculated from at least three independent experiments. All other data were analyzed by Student's *t* tests with the SAS program (SAS software; SAS Institute Inc.). Significance of differences between experimental groups was accepted at a *P* value of <0.05.

3. Results

3.1. CabA is required for the resistance of biofilms to decontamination strategies in vitro

To examine the role of CabA in the resistance of *V. vulnificus* biofilms to decontamination strategies *in vitro*, two different assays were performed using the isogenic *cabA* mutant. Once the biofilms of the wild type and *cabA* mutant formed on the test tube surfaces, they were detached by vibration, and the residual biofilms were quantified by CV staining assays (Fig. 1A). The residual biofilm of the wild type decreased more gradually by vibration than that of the *cabA* mutant. After 80 s of vibration, approximately 60% of the wild-type biofilm remained, whereas approximately 40% of the *cabA* mutant biofilm remained, indicating that the *cabA* biofilm is not as resistant as the wild-type biofilm to detachment (Fig. 1A). Similarly, the *cabA* mutant biofilm revealed reduced resistance to NaOCl disinfection compared to the wild-type biofilm. After 7 min of NaOCl treatment, approximately 70% and 50% of the wild-type and *cabA* mutant biofilm remained, respectively, (Fig. 1B). Complementation with a functional *cabA* gene (pYM1109) restored the resistance of the *cabA* mutant biofilm to levels comparable to those of the wild-type biofilm (Figs. 1A and B). Therefore, the reduced resistance of the *cabA* mutant resulted from the inactivation of functional *cabA* rather than any polar effects on genes downstream of *cabA*. These combined results demonstrated that CabA is required for the optimum resistance of *V. vulnificus* biofilm to decontamination strategies.

3.2. The cabA expression is induced in the cells bound to oyster

There are several lines of evidence that *V. vulnificus* adheres to oyster as the primary route of infection and forms biofilms to survive in hostile environments to reach a concentrated infectious dose (Froelich and Oliver, 2013; Paranjpye et al., 2007). This prompted us to examine whether the *cabA* expression is induced when *V. vulnificus* binds to oyster. Therefore, levels of the *cabA* mRNA of the wild-type cultures grown with different conditions were analyzed (Figs. 2A and B). The levels of the *cabA* transcript in the planktonic cells grown with oyster meat or shells were not significantly different from those in the planktonic cells grown with VFMG. In contrast, the cells of the biofilm formed on the surface of oyster meats or shells expressed about 4- and 10-fold higher levels of *cabA* compared to the planktonic cells grown with VFMG. This up-regulation of the *cabA* expression in biofilm cells led us to hypothesize that CabA could contribute to the development of *V. vulnificus* biofilms on the surface of oyster meat and shells.

3.3. CabA contributes to biofilm formation on oyster

To examine this hypothesis, the capabilities of the wild type and isogenic *cabA* mutant to form biofilms on oyster were compared. For this purpose, oyster meat and shells were inoculated with the wild type and *cabA* mutant and the resulting biofilms were quantified. When the biofilm cells on the oyster meats were enumerated, the number of the *cabA* mutant biofilm cells were about 2-fold less than that of the wild type (Fig. 3A). Similarly, the *cabA* mutant biofilms on the oyster shells revealed 2-fold reduced amounts of cells compared to the wild-type biofilms (Fig. 3B). No *V. vulnificus* cells appeared from the oyster meats or shells incubated without artificial inoculation of *V. vulnificus* (data not shown). The reduced amounts of biofilms formed by the *cabA* mutant suggested that CabA contributes to the development of the *V. vulnificus* biofilms on oyster meat and shells. It is noteworthy that the population of both wild-type and *cabA* mutant biofilms was about 10-fold greater on the oyster meat than the shells (Figs. 3A and

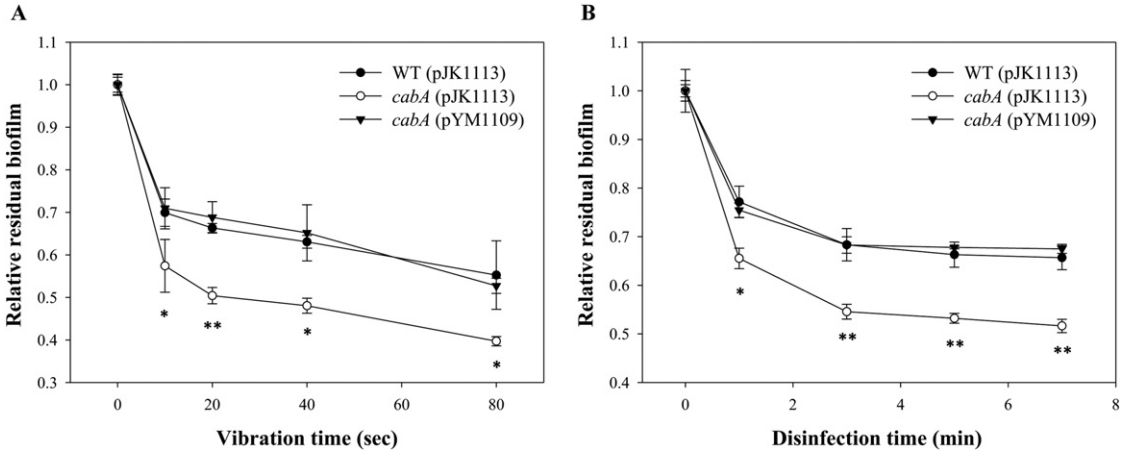

Fig. 1. Effects of the *cabA* mutation on the detachment and disinfection of biofilms *in vitro*. Biofilms of the strains, which were grown on test tubes, were either (A) detached by vibration at the speed of 1200 rpm using a micro mixer (Confido-S20, FINEPCR) or (B) disinfected by treatment with 10 ppm of NaOCl solution for various times as indicated. The residual biofilms were stained with CV and the CVs eluted with 100% ethanol were measured at an absorbance of 570 nm (A_{570}). The ratio of absorbance at the time points to the absorbance at 0 s (or 0 min) was defined as the relative residual biofilm. Ampicillin and kanamycin (100 μg/ml for each) were used to maintain plasmids in the strains and 0.01% (w/v) of arabinose was used to induce the expression of *cabA*. *, $P<0.05$; **, $P<0.005$ relative to the wild type. Error bars represent the SD. WT (pJK1113), wild type; *cabA* (pJK1113), *cabA* mutant; *cabA* (pYM1109), complemented strain.

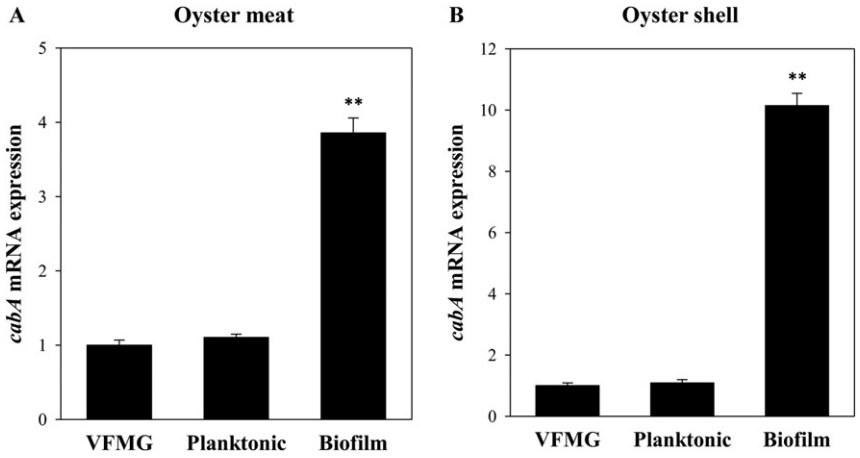

Fig. 2. Induction of *cabA* expression in the cells bound to oyster. Biofilms of the wild type were grown on the surface of oyster meat (A) or shells (B). The planktonic and biofilm cells were harvested separately and then used to isolate total RNAs. The *cabA* mRNA levels in the total RNAs were determined by qRT-PCR analyses, and the *cabA* mRNA levels in the planktonic cells grown with VFMG (control) of each sample were presented as 1. **, $P < 0.005$ relative to the *cabA* mRNA levels in the planktonic cells grown with VFMG. Error bars represent the SD.

B). One possible explanation for this variation is that the biofilm cells on the oyster meat grow faster to reach higher concentrations than those on the shells where nutrients are probably not sufficient to support such growth.

3.4. CabA limits detachment of biofilms on oyster

To further investigate the role of CabA in *V. vulnificus* biofilm developed on oyster, the resistance of the wild-type and *cabA* mutant biofilms to the decontamination strategies was compared. For this purpose, cells which were detached from biofilms after vibration for various periods were enumerated and converted to the residual biofilm cells (Fig. 4). When the biofilms on oyster meat were vibrated, more cells were detached from the *cabA* mutant biofilms than the wild-type biofilms (Fig. 4A). Most of the biofilm cells of the *cabA* mutant were detached after vibration for 20 s. In contrast, the wild-type biofilm cells were detached more gradually and most of the detachment had occurred after vibration for 60 s (Fig. 4A). Similarly, the biofilm cells of the *cabA* mutant on the oyster shells were detached more rapidly by vibration than those of the wild type (Fig. 4B). Most cells were detached from the *cabA* mutant biofilm after vibration for 60 s, whereas 15% of the population of the wild-type biofilm cells still remained (Fig. 4B). From these results, it is apparent that the biofilms of the wild type were more robust and thus resistant to the detachment by vibration than those of the *cabA* mutant. Formation of more robust biofilms by the wild type suggested that CabA has effects on the structure as well as the amount of biofilms formed on oyster.

3.5. CabA limits disinfection of biofilms on oyster

In addition to physical detachment, many chemicals are adopted to disinfect the biofilms (Srey et al., 2013). To examine the possible role of CabA in the resistance of biofilms to disinfection, the wild-type and *cabA* mutant biofilms on the surface of oyster meat and shells were disinfected with NaOCl, and cells that survived in the biofilms were enumerated (Fig. 5). When biofilms on the surface of oyster meat were disinfected, the survival of the *cabA* mutant biofilm cells decreased more rapidly than the wild-type biofilm cells. After 10 min disinfection, approximately 30% of the *cabA* mutant biofilm cells survived, whereas survival of approximately 60% the wild-type biofilm cells was observed (Fig. 5A). Similarly, when biofilms on the surface of oyster shells were disinfected, approximately 70% and 25% of the wild-type and *cabA* mutant biofilm cells survived, respectively (Fig. 5B). The results demonstrated that the biofilms of the wild type on oyster were more adverse and resistant to chemical disinfection than those of the *cabA* mutant. Taken together, the combined results led us to conclude that CabA

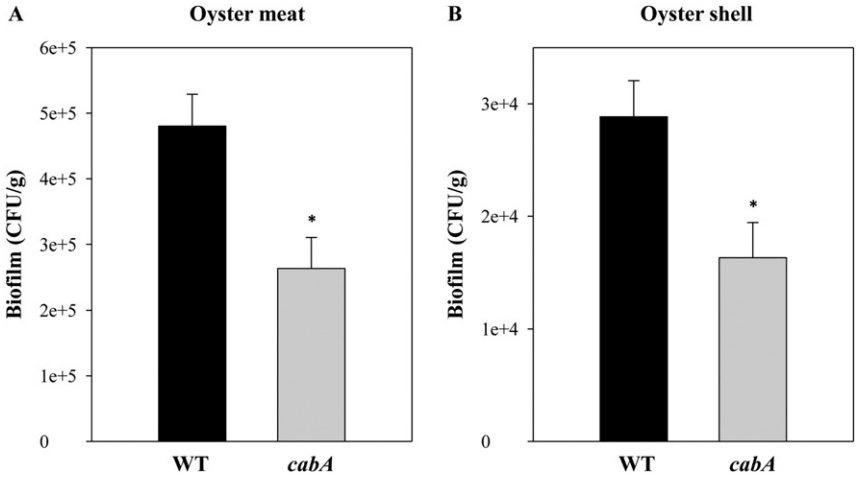

Fig. 3. Effects of *cabA* mutation on the formation of biofilms on oyster. Biofilms of the strains were grown on the surface of oyster meat (A) or shells (B). Once planktonic cells were gently removed, the biofilm cells were enumerated as CFUs on LBS agar plates containing 100 U/ml of polymyxin B. The number of biofilm cells were normalized to CFUs per gram of oyster (meat or shell, respectively). *, $P < 0.05$ relative to the wild type. Error bars represent the SD. WT, wild type; *cabA*, *cabA* mutant.

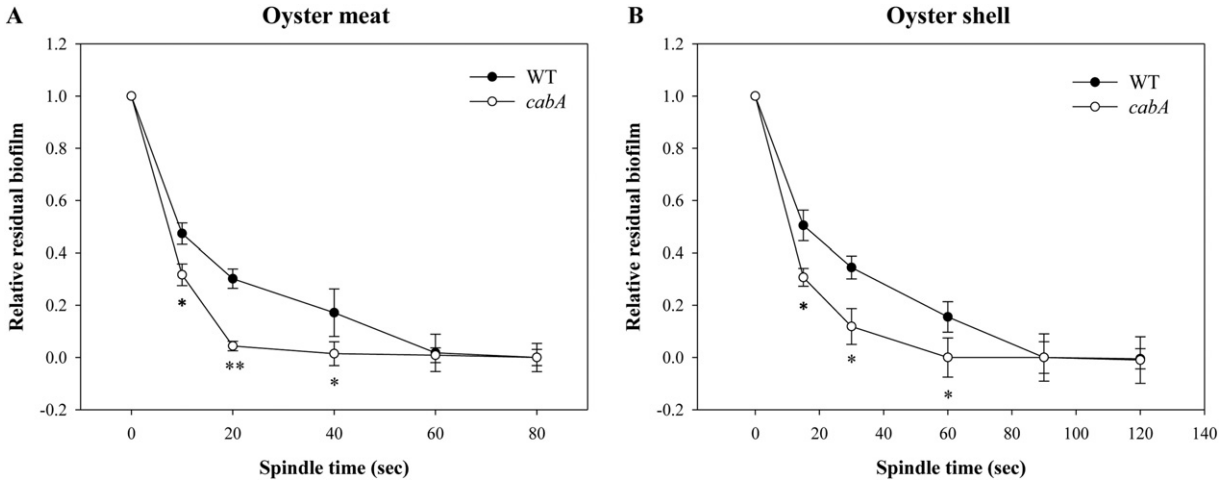

Fig. 4. Effects of *cabA* mutation on the detachment of biofilms on oyster. Biofilms of the strains, which were grown on the surface of oyster meat (A) or shells (B), were detached by vibration at the speed of 7000 g using the Spindle (Kim et al., 2012) for various times as indicated. The detached biofilm cells were enumerated as CFUs on LBS agar plates containing 100 U/ml of polymyxin B. The residual biofilm cells were calculated by subtracting the numbers of detached cells from the total number of biofilm cells (A, for meat) or from the number of biofilm cells detached at 90 s (B, for shells), respectively. The relative residual cells were then determined by dividing the number of residual biofilm cells at the time points by the total number of biofilm cells (A, for meat) or by the number of biofilm cells detached at 90 s (B, for shells), respectively. *, $P < 0.05$; **, $P < 0.005$ relative to the wild type. Error bars represent the SD. WT, wild type; *cabA*, *cabA* mutant.

enables *V. vulnificus* to build robust biofilms on oyster as well as *in vitro*, and thereby contributes to withstanding decontamination strategies such as physical detachment and chemical disinfection.

3.6. CabA consolidates biofilm structures on oyster

To get a better understanding of the mechanisms by which CabA renders the *V. vulnificus* biofilms recalcitrant, biofilms of the wild type and *cabA* mutant were grown on the oyster shells and their structures were examined using SEM before and after detachment by vibration (Fig. 6). Before the detachment, the wild type formed thick and more structured biofilms on the oyster shells along with filamentous materials connecting bacterial cells in the extracellular matrix. In contrast, the *cabA* mutant cells were scattered and unstructured without any extracellular filaments binding the cells together, albeit the occasional appearance of few bacterial cell clusters. After the detachment by vibration for 30 s, the residual biofilms of the wild type are still substantial and filaments that connect the bacterial cells to each other were still visible. In contrast, most of the *cabA* mutant biofilms were removed and only few cells were left nearly bare. The results suggested that CabA contributes to the structural integrity of the *V. vulnificus* biofilms presumably by forming filaments in the matrix and thereby allows the biofilms to survive under decontamination strategies.

4. Discussion

EPM forms the framework for the three-dimensional architectures of biofilms (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004). The major components of the EPM are extracellular polysaccharides (EPS), proteins, and DNA (eDNA), which are distributed between the cells in a non-homogeneous pattern (Flemming and Wingender, 2010). Diverse extracellular polysaccharides are produced by *V. vulnificus* and their functions in the development of biofilms are well characterized (Guo and Rowe-Magnus, 2011; Kim et al., 2007; Kim et al., 2009; Lee et al., 2013), and their structural and regulatory genes involved in the *V. vulnificus* EPS production and, in turn, biofilm formation have been extensively studied (Garrison-Schilling et al., 2014; Grau et al., 2008; Guo and Rowe-Magnus, 2010, 2011; Kim et al., 2009; Kim et al., 2011). However, compared to EPS, little is known about the non-EPS components of the *V. vulnificus* EPM and their expression patterns.

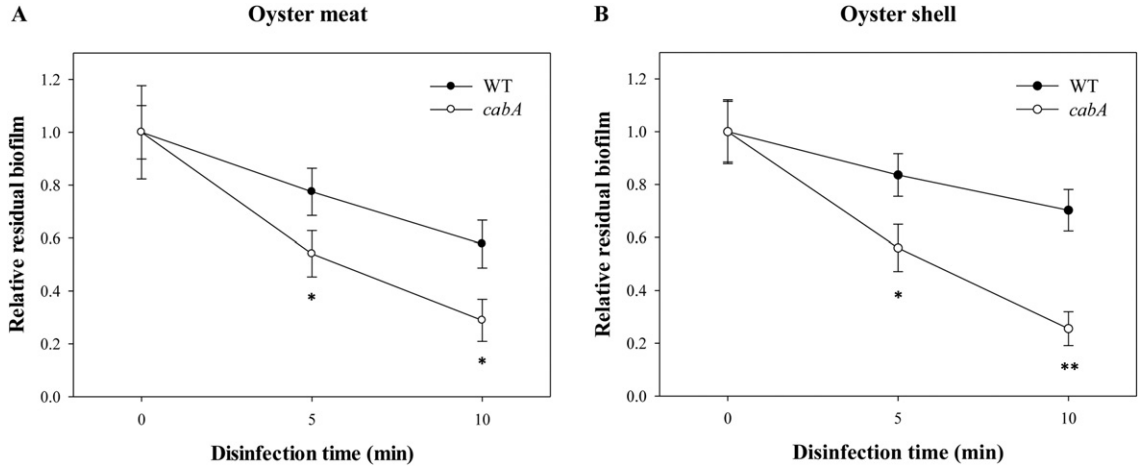

Fig. 5. Effects of *cabA* mutation on the disinfection of biofilms on oyster. Biofilms of the strains, which were grown on the surface of oyster meat (A) or shells (B), were disinfected by incubating with 10 ppm of NaOCl solution for various times as indicated. Cells that survived in the residual biofilm were enumerated as CFUs on LBS agar plates containing 100 U/ml of polymyxin B. The ratio of CFUs survived at the time points to the number of CFUs at 0 s was defined as the relative residual biofilm. *, $P < 0.05$; **, $P < 0.005$ relative to the wild type. Error bars represent the SD. WT, wild type; *cabA, cabA* mutant.

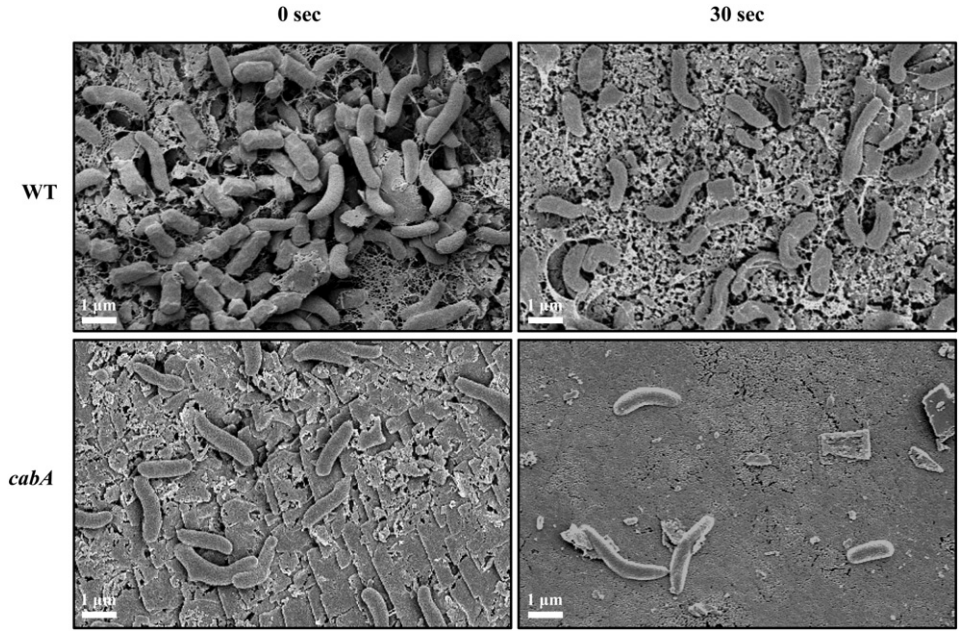

Fig. 6. Electron micrographs of biofilms on the oyster shell. Biofilms of the strains, which were grown on oyster shells, were detached by vibration at the speed of 7000 g using the Spindle for 30 s as indicated. The residual biofilm cells before (0 s) and after (30 s) detachment were fixed, dehydrated, coated with platinum, and visualized using SEM (Supra 55VP, Zeiss) at a 10,000× magnification. Bars, 1 μm; WT, wild type; *cabA*, *cabA* mutant.

Recently, CabA, an extracellular protein that is distributed throughout the *V. vulnificus* biofilm matrix, has been identified and characterized *in vitro* (Park et al., 2015). CabA is a calcium binding protein, alters its conformation in a calcium-dependent manner, and contributes to biofilm formation in microtiter plates (Park et al., 2015). The present study demonstrated that the *cabA* expression was up-regulated upon the binding of *V. vulnificus* to oyster (Fig. 2). Furthermore, the wild type developed greater amounts of biofilms on oyster meat and shells compared to the *cabA* mutant (Figs. 3A and B), validating that CabA contributes to biofilm formation of *V. vulnificus* not only *in vitro* but also on oyster.

Standard practices such as the use of antimicrobial agents and oxidizing agents, which were developed to decontaminate planktonic bacterial cells, are not sufficient to eradicate bacterial cells in biofilms (Jahid and Ha, 2012). Therefore, there is increasing demand for development of new strategies to replace or to complement the standard practices (Meyer, 2003). Among them, inhibition of the optimum function and/or the biogenesis of the extracellular matrix components has emerged as a novel strategy to prevent formation or to promote disassembly of biofilms. Accordingly, enzymes such as amylase, dextranase and alginate lyase that are able to degrade matrix EPS were used to suppress formation of biofilms by various pathogens and to enhance susceptibility of the established biofilms of the pathogens to antimicrobial agents (Alkawash et al., 2006; Ivanova et al., 2015; Li et al., 2012). Similarly, DNases that destabilize biofilms and increase antimicrobial susceptibility by degrading eDNA were proven as useful tools in biofilm control (Okshevsky et al., 2015). Alternatively, small-molecule compounds inhibiting the biogenesis of curli, which are functional amyloid fibers and components of the uropathogenic *Escherichia coli* EPM, were identified and shown to prevent biofilm formation by the pathogen (Cegelski et al., 2009).

Although it was previously apparent that CabA is essential for the development of well-structured mature biofilms of *V. vulnificus* in flow cells (Park et al., 2015), there was a lack of understanding regarding its role in the resistance of the resulting biofilms to decontamination strategies. Results presented here show the wild-type biofilm was more resistant to vibration detachment and NaOCl disinfection than the *cabA* mutant biofilms (Figs. 1, 4, and 5), indicating that CabA enhances structural integrity of the biofilms. Microscopic examination demonstrated that the bacterial cells in the wild-type biofilms were connected together by filaments that were not apparent in the *cabA* mutant biofilms (Fig. 6). These observations suggest that CabA strengthens the structure of biofilms by mostly, if not solely, forming filaments that aid *V. vulnificus* cells in adhering to surfaces and thus assemble together. This leads us to propose that CabA is a good target for detachment or disassembly of *V. vulnificus* biofilms on oyster. As such, this discovery can be used to establish environments which can minimize the expression of *cabA* and/or inhibit the activity of CabA during transportation, processing and storage of oyster. In other ways, the combinatory use of the small molecules inhibiting the synthesis or activity of CabA could increase the effectiveness of the current chemical disinfectant (or physical detachment) to eliminate the pathogen from oyster in the postharvest process and thereby ensure the safety of raw oyster. Accordingly, experiments to identify the small molecules are underway.

In summary, this study demonstrated that CabA, an extracellular matrix protein of the *V. vulnificus* biofilms, is induced upon binding of the pathogen to oyster. The *cabA* mutant was significantly defective in the development of biofilms on the oyster meat and shells. Furthermore, compared to the wild-type biofilm, the *cabA* mutant biofilm was more susceptible to detachment by vibration and disinfection by NaOCl on oyster as well as *in vitro*. It appears that CabA contributes to the formation of filaments in the biofilm matrix where the filaments connect bacterial cells together to build robust biofilms that are resistant to decontamination strategies. Consequently, the results of this study will be useful to design an effective postharvest process program to reduce the pathogen in oyster and further ensure the safety of oyster.

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