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Bacteriology

Membrane vesicle protein PagC as a novel biomarker for detecting pathogenic *Salmonella* in the viable but not culturable state

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ABSTRACT. The viable but non-culturable (VBNC) state is a remarkable survival mechanism in which cells exist in a physiologically inactive state. Bacteria in the VBNC state do not form colonies, and thus, are difficult to detect using colony-based methods. As a result, VBNC bacteria are potentially virulent and can cause widespread contamination during food production. In the present study, we reported a novel biomarker, the membrane vesicle protein PagC, for the detection of VBNC *Salmonella*. *Salmonella* cells were chemically induced into the VBNC state by H_2O_2 treatment. The bacterial cells retained their shapes but were observed to release numerous membrane vesicles, which were accompanied by a transient PagC overexpression. Immunoblotting was performed to detect PagC in pathogenic strains, including *Salmonella* Enteritidis and *S.* Typhimurium, which are harmful and known to cause food-borne gastroenteritis in humans and other animals. Therefore, our findings demonstrated the potential use of PagC as a biomarker for the detection of VBNC *Salmonella* in food production.

KEY WORDS: PagC, *Salmonella*, VBNC, vesicle protein

The viable but non-culturable (VBNC) state is a unique but effective survival strategy that is observed in many bacterial cells when subjected to unfavorable environments [9, 16]. The VBNC state has been generally considered a dormant state. However, VBNC cells were shown to be more metabolically active than previously thought. Although VBNC bacteria do not divide or form colonies, they are alive and maintain the biological processes that are required to sustain life. VBNC bacteria have intact cell membranes and undergo protein synthesis and respiration [2, 24]. Previous studies have demonstrated that a broad range of pathogenic bacteria can enter the VBNC state. For instance, the VBNC states of human pathogens, such as *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Streptococcus faecalis*, and *Micrococcus flavus*, have been observed in drinking water [3]. In a previous study, Cook *et al*. confirmed that *Campylobacter jejuni* and *Escherichia coli* enter the VBNC state during unfavorable conditions [4]. The VBNC state of the hemorrhagic *E. coli* O157:H7 EHEC has also been reported [5]. Current microbiological detection is primarily based on colony formation on enriched agar. However, VBNC bacteria are unculturable and can escape detection, making them potentially virulent once the environment turns favorable [17]. Several previous studies have specifically investigated food-borne pathogens in the VBNC state, particularly within the genus *Salmonella*.

The VBNC state of *Salmonella enterica* serovar Enteritidis was first described by Roszak *et al* [18] in 1984 and was followed by similar observations in *S.* Typhimurium by Turpin *et al* [21]. In 1999, a severe food poisoning outbreak occurred in Japan, which resulted in over 1,500 patients being hospitalized, along with further diffuse outbreaks nationwide. The outbreak was confirmed to be primarily caused by undetected *Salmonella* serotype Oranienburg that contaminated dried squid products and eventually proliferated exponentially upon entry into the human body [19]. Thus, there is an urgent need for alternative methods for the detection of VBNC *Salmonella*. Systematic evolution of ligands by exponential enrichment (SELEX) was previously introduced as a method of detecting VBNC *Salmonella* [12]. However, a single round of detection via SELEX is relatively laborious. Additional methods that rely on the detection of distinct biomarkers can simplify the process and reduce the costs for VBNC screening.

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When the bacterial envelope is subjected to stressors, membrane vesicles are formed to relieve the physical pressure [13] in a process similar to the induction of the VBNC state. In the present study, we focused on the membrane protein PagC, one of the protein components of membrane vesicles that are released by *Salmonella* cells as an essential defense mechanism [11, 22]. PagC was originally named PhoPQ-activated gene [8] and functions by upregulating the expression of more than 40 genes required for virulence. In addition, PagC is involved in the biological activities of *S.* Enteritidis under unfavorable conditions, including growth under low Mg^{2+} concentrations, and mediates resistance to antimicrobial peptides, bile salts and acidic pH [6, 7, 20, 23]. PagC has been reported to be upregulated under stressful conditions, such as low pH or low Mg^{2+} concentrations [22]. PagC is involved in the intracellular survival of *Salmonella* inside macrophages by inhibiting bacterial cell division and prolonging the cell cycle [11]. This suggests that PagC acts by inducing *Salmonella* cells to eventually enter VBNC state under unfavorable environmental conditions. We therefore examined the protein expression of PagC in response to H_2O_2 treatment as previously reported by Morishige *et al.* [9]. Our results showed that although different *Salmonella* strains had similar PagC expression levels, PagC is significantly overexpressed in the VBNC state, thereby highlighting the practical value of PagC as a biomarker for the detection of VBNC in *Salmonella*.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals

S. Enteritidis zSE1 and *S.* Typhimurium st1wt [10] were used in this study. Both *Salmonella* are wild-type strains and are genetically intact. Strains were cultured in Trypto-Soya Broth (TSB) (Nissui Chemicals, Tokyo, Japan) adjusted to pH 7.3 at 37°C. Bacteria were treated with H_2O_2 (Wako Pure Chemicals, Osaka, Japan) to chemically induce the VBNC state.

Artificial induction of the VBNC state

To identify potential biomarkers for detecting VBNC *Salmonella*, cells were induced to the VBNC state. H_2O_2 was selected as the chemical inducer because of its short induction period of several hours and high effectiveness as a stress factor [14]. *Salmonella* cells were grown in TSB at 37°C until reaching the early stationary phase. Cell cultures were diluted to 10^6 cfu/m*l*. Next, 1 m*l* of diluted culture was washed twice with phosphate-buffered saline (PBS) via centrifugation (15,000 × g) at room temperature (20°C) for 5 min. Cells were resuspended in 1 m*l* of TSB. TSB (9 m*l*) containing 3 mM H_2O_2 was added to the suspension to obtain a final volume of 10 m*l*. The resulting mixture was cultivated at 37°C with shaking at 120 strokes per min. Samples were collected and washed as described above every 20 min for subsequent cultivation on agar plates. Colony counting was performed the next day.

Assessment of H_2O_2 induction via viability testing

Viability testing of VBNC-induced *Salmonella* cells was performed using Bacterial Viability Kits (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to the manufacturer's protocol. Briefly, after 2 hr of induction in the H_2O_2 medium, *Salmonella* cells were collected by centrifugation (10,000 × g, 15 min, room temperature) and washed twice with 0.85% NaCl solution. Cells were then stained with SYTO9 and propidium iodide according to the manufacturer's instructions. After 15 min of incubation in the dark at room temperature, cell viability was assessed using a fluorescent microscope (FSX100, Olympus, Tokyo, Japan).

Confirmation of PagC release from VBNC Salmonella cells

Immunoblotting was performed to examine PagC expression in VBNC *Salmonella* cells. Samples were prepared via centrifugation of the VBNC cultures (3,000 × *g*, 15 min, RT), washing with PBS, and resuspension with PBS. Normally cultured cells were prepared following the same procedure as that of the control samples. Protein samples collected from the culture supernatant were confirmed via SDS-polyacrylamide gel electrophoresis using the appropriate molecular size markers. Samples were loaded onto polyacrylamide gels and blotted onto PVDF membranes (EMD Millipore, Burlington, MA, U.S.A.). Next, membranes were incubated with anti-PagC rabbit antibodies, which were generated from the previous study of Yamamoto *et al.* [6]. Briefly, purified PagC fusion proteins were eluted with an imidazole step gradient. Aliquots of the peak fraction were then dialyzed against dialysis buffer [10 mM PBS, 8 M urea (pH 7.4)] and used to immunize rabbits. Anti-rabbit IgG antibodies [horseradish peroxidase (HRP)-conjugated] (Santa Cruz Biotechnology Inc., Paso Robles, CA, U.S.A.) were used to determine the PagC protein levels. Immunoblotting results were evaluated with a luminol-based chemiluminescence (Nacalai Tesque, Kyoto, Japan) assay.

RESULTS

Bacterial colony formation after H_2O_2 treatment

We performed colony counting on agar plates to assess the colony formation abilities of *S.* Enteritidis and *S.* Typhimurium in response to H_2O_2 treatment. Both strains showed a descending streak of colony formation over time after treatment with H_2O_2. No colonies were formed after 2 hr of H_2O_2 treatment (Fig. 1).

Viability testing

Both *Salmonella* strains were unable to form colonies after H_2O_2 treatment. However, these results could be caused by cell death. To verify that the lack of colony formation is caused by induction of the VBNC state, we conducted viability testing via fluorescent staining. Propidium iodide can only diffuse across damaged cell membranes, whereas SYTO9 can enter both intact and

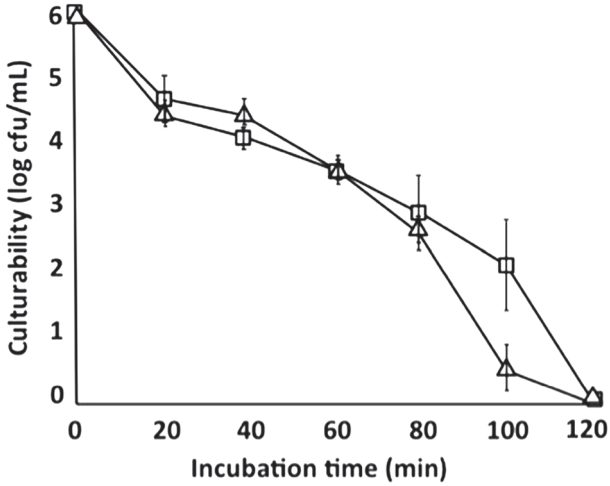

Fig. 1. Relative culturability of *Salmonella* in agar plates after H_2O_2 treatment. Both *S.* Enteritidis (square) and *S.* Typhimurium (triangle) showed decrease on colony number. The experiment was performed in triplicate. Vertical lines denote the standard deviation.

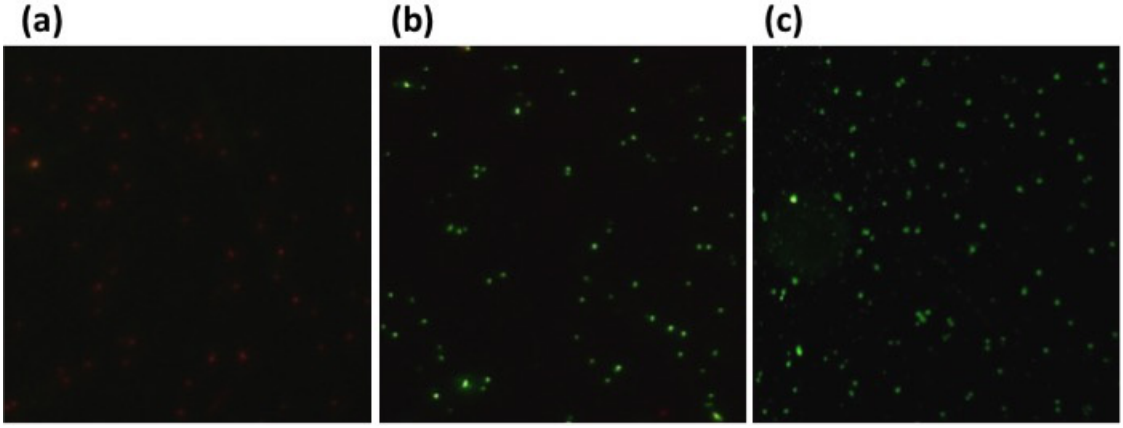

Fig. 2. Viability testing of VBNC *S.* Enteritidis. (a) Cells killed with 70% isopropanol appear red in color; (b) Untreated live cells appear green; (c) Cells treated with H_2O_2 for 2 hr also appear green.

damaged cell membranes. Results showed that cells treated with 70% isopropanol were red in color (Fig. 2a), which indicated the presence of dead cells (negative control). By contrast, untreated living cells were used as positive control, appeared green (Fig. 2b). Cells collected after 2 hr of H_2O_2 treatment showed the same greenish (Fig. 2c) color as those of living cells. These results indicated that H_2O_2-treated cells were still alive but were incapable of forming colonies.

Morphology of Salmonella cells in the VBNC state

Salmonella cells in the VBNC state were observed using a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan). In contrast to untreated normal cells (Fig. 3a), cells subjected to 24 hr of VBNC induction released numerous membrane vesicles (Fig. 3b), which is known as a survival mechanism of bacterial cells subjected to certain stressors like H_2O_2. These results were consistent with a previous study that used H_2O_2 as a stress factor [14].

Immunoblotting

PagC expression following VBNC induction was examined by immunoblotting (Fig. 4). PagC expression in *S.* Enteritidis cells did not significantly change within 24 hr, although the initial PagC levels were slightly higher (Fig. 4a). By contrast, PagC expression was significantly upregulated in *S.* Typhimurium after several hours of VBNC induction and further increased substantially within 24 hr (Fig. 4b). PagC levels in *S.* Enteritidis were similar level between days 0 and 3 but increased on day 7 (Fig. 4c). PagC expression steadily increased when the sampling period was prolonged for up to 7 days (Fig. 4c). Despite minor differences in expression, both strains released considerable amounts of PagC in the VBNC state. Experiments were performed in triplicate and showed consistent results.

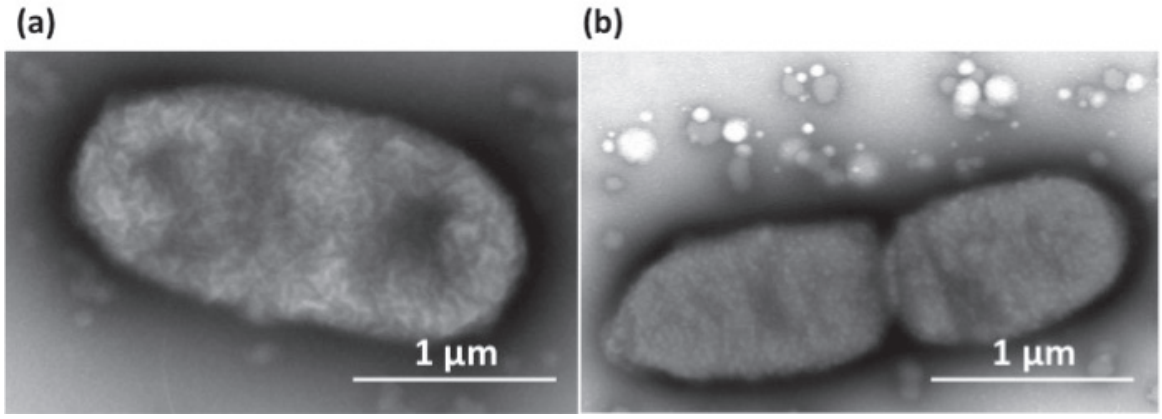

Fig. 3. Morphologies of *Salmonella* cells. (a) Normal *S.* Enteritidis cells; (b) VBNC cells induced with H_2O_2 for 24 hr.

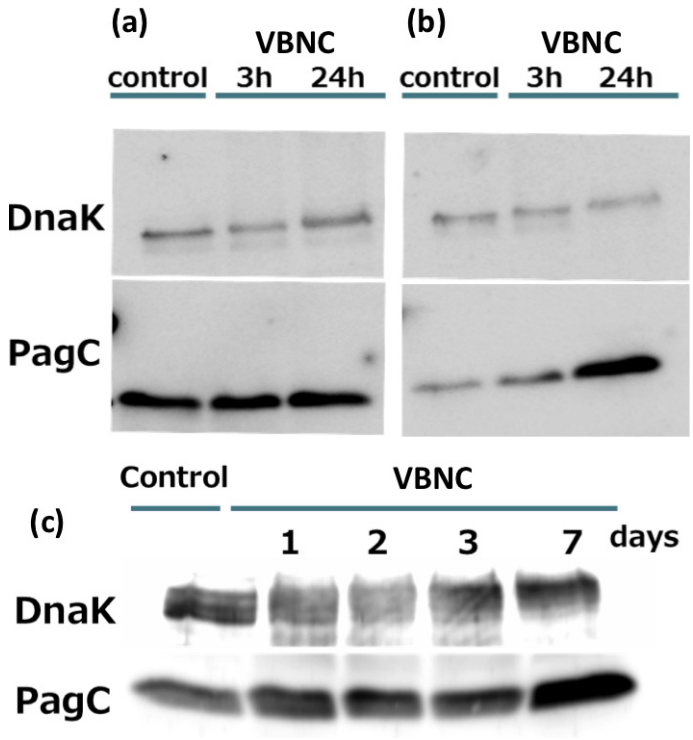

Fig. 4. PagC expression was assessed by immunoblotting. PagC samples were collected from (a) *S.* Enteritidis and (b) *S.* Typhimurium after 24 hr. PagC expression in *S.* Typhimurium increased over time, whereas PagC levels in *S.* Enteritidis did not change. (c) *S.* Enteritidis showed PagC upregulation over time for 7 days. Experiments were performed in triplicate and showed consistent results.

DISCUSSION

Bacteria in the VBNC state are physiologically inactive but retain their virulent properties and could be resuscitated by passing through a host animal including humans [1], thereby demonstrating that VBNC bacteria are potentially pathogenic. Many commercial decontamination treatments in food production cannot eliminate and even induce bacteria to enter the VBNC state. For example, freezer pasteurization of fruit juice was found to induce the VBNC state in *S.* Typhimurium and *E. coli* O157:H7 [15]. In addition, lack of proper detection methods further increases the risk of exposure to contaminated food products. Hence, there is an urgent need to develop reliable methods to assess VBNC bacteria and their viability.

PagC is a component of membrane vesicles and acts by inhibiting the proliferation of *Salmonella* within macrophages [14]. In the present study, focused on the well-known bacterial pathogen *Salmonella*, which causes food-borne gastroenteritis in humans.

Microscopic observations confirmed the release of membrane vesicles during the H_2O_2-induced VBNC state (Fig. 3), which is known as a survival strategy of *Salmonella* when subjected to environmental stress, and is accompanied by PagC expression. Interestingly, increase in PagC expression in *S.* Typhimurium st1wt was considerably faster than that observed in *S.* Enteritidis zSE1, which could serve as a basis for distinguishing *Salmonella* pathogens at the species level. Our study is the first to report the association between PagC protein and the VBNC state in *Salmonella*. Thus, we propose the use of PagC as a biomarker for the rapid detection of VBNC *Salmonella* during food production.

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