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Role of in vivo passage on the environmental adaptation of enterohemorrhagic *Escherichia coli* O157:H7: Cross-induction of the viable but nonculturable state by osmotic and oxidative stresses

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Abstract

In an enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 outbreak caused by salted salmon roe that occurred in Japan, 1998, a food isolate (F2) was NaCl-resistant and a patient isolate (P5) was sensitive to NaCl. We show here that hydrogen peroxide, like NaCl, induced a significant loss of culturability in P5. The BacLight assay suggested that the EHEC O157:H7 entered a viable but nonculturable (VNC) state. We used the passage through mice in an attempt to model this transition in phenotype. Mouse-passaged isogenic variants of F2 became NaCl- and oxidation-sensitive, entered the nonculturable state in response to either of these stresses, and could be resuscitated by sodium pyruvate. Since the expression of RpoS in response to these stresses correlated with the isolates' culturabilities, we concluded that in vivo passage negatively modulated RpoS expression, and the subsequent stress exposure induced the VNC state in the EHEC O157:H7 isolates.

Keywords: EHEC O157; VNC; Hydrogen peroxide; NaCl; RpoS

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes diarrhea, hemolytic uremic syndrome (HUS), or asymptomatic infection in humans and animals. This pathogen is often isolated from various non-host environments [1], where the bacteria have been excreted from natural mammalian hosts, including humans. Once excreted from the host, *E. coli* cells face various environmental stresses, such as changes in osmolarity, lowered pH, UV-C radiation, nutrient starvation, and heat shock [2–4]. Under these conditions, bacteria protect their viability by entering the viable but nonculturable (VNC) state. The VNC state is one of physiological dormancy, in which metabolic activities and membrane permeabilities are retained, but these cells hardly grow at all in commercial enrichment media [5,6]. The state of dormancy seems to mask the actual number of viable cells, which should be taken into account in the field of microbial inspection. Many reports on the induction of the VNC state in *E. coli*, *Salmonella*, *Campylobacter*, and *Vibrio* spp. have described its occurrence in aquatic environments, such

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as seawater, ground water, and rivers [7–10]. However, relative to the knowledge about microbial ecology, little is known about the effects of this physiological dormancy on food safety.

It has long been believed that food treatments, including lowering the pH, desiccation, and increasing the osmolarity with NaCl, are bacteriostatic for food-borne pathogens, but it is now clear that these conditions can induce the VNC state in food-borne bacteria. Since the VNC bacterial cells can be resuscitated from dormancy by the appropriate conditions [11–13], the dormant cells can pose a health risk. For example, a diffuse outbreak of EHEC O157:H7, caused by salted salmon roe, occurred in 1998 in Japan. The most probable number (MPN) method of assessing how many cells caused the infection estimated that 0.75–1.5 viable EHEC O157:H7 cells were sufficient [14]. This number was considered to be very low for infection [15], and therefore we suspected that large numbers of EHEC O157:H7 might have entered the VNC state in the food. This hypothesis was proved by re-estimating the numbers of bacterial cells from their membrane integrity, cell elongation, and pathogenicity for mice [16]. The induction of the VNC state in EHEC O157:H7 in food suggested that the EHEC O157:H7 might be induced to enter the VNC state by other stresses, including food treatments for disinfection and storage. To clarify what stresses might induce the EHEC O157:H7 to enter the VNC state, we first investigated the culturability and viability of the pathogen under increased NaCl, hydrogen peroxide, UV-C radiation, acid, and heat stresses. In addition, the effects of in vivo passage on changes in the bacterial susceptibility to environmental stresses, the ability of VNC cells to be resuscitated, and the expression of RNA polymerase sigma S (RpoS) in EHEC O157:H7 under stress conditions were investigated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

EHEC O157:H7 strains of food and patient origin from the 1998 outbreak were renamed "F2" and "P5"; they were called strains 2 and 5 in our previous study [16]. L-broth was used as the liquid media. To measure the most probable numbers (MPN), buffered peptone water and trypticase soy broth (Beckton Dickinson) were used as the pre-enrichment and growth media, respectively.

2.2. Culturability of EHEC O157:H7 under stress conditions

Approximately 10^8 strain F2 and P5 cells grown in stationary phase were suspended in 10 ml of sterile phosphate buffered saline (PBS, pH 7.4), and then heat shocked (56 °C for 6 h), or exposed to UV irradiation (0.05–12.8 J/cm^2 for 15 s to 64 min). Osmotic shock was studied by incubating the bacterial cells in a 13% NaCl solution at 37 °C. To investigate survival under oxidative and acidic stress, the same numbers of bacteria as above were incubated in either 10 ml of PBS containing 0.05% hydrogen peroxide (Wako Chemicals, Osaka, Japan) or low-pH PBS (pH 3.0 or 4.0 adjusted with acetic acid) at 37 °C. From these incubated suspensions, 0.1-ml aliquots and serial dilutions of each were spread on trypticase soy agar (TSA, Becton Dickinson) to quantify the culturable cells. Duplicate experiments were performed, and the data were described as the means of three determinations in each experiment. In cases in which the culturabilities of strains F2 and P5 were significantly different, bacterial viability was monitored by quantifying the fluorescent staining obtained by the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes Inc., Eugene, Oregon) [7]. 1500 cells were randomly selected from eight separate microscopic fields, and the numbers of live (green fluorescence) and dead (red fluorescence) cells were calculated.

2.3. Isolation and phenotypic test of NaCl-sensitive isogenic variants of strain F2

Female BALB/c mice ($n = 3$) aged 6–8 weeks (purchased from SLC Japan) were inoculated with 10^8 cells of food strain F2 by oral administration. At 24 h post infection, the mice were sacrificed to collect the cecum contents aseptically. Samples of 0.1 g were suspended in sterile PBS, and spread on Sorbitol MacConkey agar (Eiken kagaku, Tokyo, Japan). Fifty O157:H7 colonies that were randomly isolated from each mouse were grown in L-broth for 20 h, and 0.1 ml of each of these cultures was incubated in 10 ml of 13% NaCl solution for 24 h to investigate their NaCl sensitivity. The suspensions were then spread on TSA to quantify the cell survival. Strain F2 formed colonies at $>10^5$ CFU/ml and P5 isolates formed them at <10 CFU/ml (data not shown). The isolates from mice were considered to be NaCl-sensitive when survival numbers were lower than 10 CFU per 0.1 ml.

2.4. Culturability, viability, and resuscitation response of EHEC O157:H7 under oxidation

NaCl-sensitive isogenic variants of strain F2, obtained by mouse passage, and designated as strains MP14, MP37, and MP62, were incubated in L-broth for 20 h, and then incubated in 10 ml PBS containing 0.05% hydrogen peroxide at 37 °C for 144 h. Their culturabilities were examined by plate count on TSA and their viability by BacLight staining, as described above. The MPN was also calculated by using three sets

of a 10-fold dilution series in buffered peptone water, followed by enrichment in trypticase soy broth at 37 °C. The resuscitation response was quantified by plate counts on TSA amended with 0.1% sodium pyruvate (Wako, Osaka, Japan).

2.5. Expression of RpoS in EHEC O157:H7 in response to osmotic and oxidative stresses

To examine the relationship between RpoS expression and the culturability of EHEC O157:H7 grown under osmotic or oxidative stress, western blotting was performed using an *anti*-RpoS rabbit antiserum. The signals were detected by chemiluminescence using ECL plus (Amersham Bioscience), and quantified using Quantity One software (Bio-Rad Laboratories).

3. Results and discussion

3.1. Stress-susceptibility testing

When EHEC O157:H7 strains F2 and P5 were incubated in 13% NaCl solution, the food strain F2 showed transiently decreased culturability and viability, although about 1.70% of the inoculum formed colonies at 144 h (Fig. 1). In contrast, the patient strain P5 showed significant susceptibility to the high osmolarity, and had formed no colonies at 72 h of incubation. However, the cellular viability remained above 80% (data not shown), as in our previous study [16]. Incubation in PBS containing 0.05% hydrogen peroxide markedly accelerated the decrease in the survival of strain P5, and it lost culturability within 6 h post-incubation. In contrast, strain F2 had a sustained culturability of 0.0203% at that time (Fig. 1). The viability of both strains gradually decreased, but green fluorescence was visible in 51.2% of the F2 colonies and 48.6% of the P5 colonies at 6 h of incubation (Table 1). This significant difference in culturability between the two strains was similar to that seen for the same strains incubated in the 13% NaCl solution.

The effects of other stresses on the survival of strains F2 and P5, including heat, UV irradiation, and acidic conditions (pH 4.0), were not significantly different (Fig. 1). Although between 2 and 6 h of incubation post-treatment strain P5 showed some recovery from the effect of lowering the pH (pH 3.0), the recovery was transient and not significant.

3.2. Passage through mice induces phenotypic changes in the sensitivity of EHEC O157:H7 to NaCl and oxidative stress

Since we expected that hydrogen peroxide stress had driven P5 into the VNC state just as NaCl osmotic stress did, we further investigated VNC induction by hydrogen peroxide. Because the phenotypic differences between the strains occurred during the passage through mice [16], isogenic variants of the food strain F2, which became NaCl-sensitive, were obtained by mouse passage, and the reactivity of these variants to oxidative stress was examined. A total of 42 out of 150 colonies became sensitive to NaCl (data not shown). Three representative NaCl-sensitive variants were randomly selected and designated as strains MP14, MP37, and MP62. When incubated in 0.05% hydrogen peroxide for 144 h, strain F2 formed the largest numbers of bacterial colonies (0.32% of the inoculum size) on TSA agar after 6 h (Fig. 2), and its colonies persisted after 120 h of incubation. With strain P5, only 0.000016% of the inoculum was detected on TSA plates after 3 h of incubation, and no colonies were found after 6 h. The mouse-passaged isogenic variants also had significantly decreased survival in hydrogen peroxide and no colonies were present on TSA plates after 6 h of incubation, as with patient strain P5, but unlike the parental food strain F2 (Fig. 2). The MPNs correlated with the plate counts, and representative mice passaged strain, MP37 cells could not be detected at 6 h of incubation (Table 1). The BacLight assay had shown that the percentages of green-fluorescing cells were 51.2% in strain F2 and 49.5% in strain P5. The green-fluorescing cells in these variants were maintained at 44.5% (MP14), 48.6% (MP37), and 44.9% (MP62), with no significant differences among the EHEC O157:H7 strains tested. These data suggested that the loss of culturability in EHEC O157:H7 in response to hydrogen peroxide might be due to induction of the VNC state, and that induction of VNC state whether by osmotic or oxidative stress, might occur through essentially the same mechanism.

3.3. Sodium pyruvate promotes the recovery of EHEC O157:H7 under osmotic and oxidative stress

The predicted viability of EHEC O157:H7 strains F2 and P5 under oxidative stress was investigated using plate counts on TSA supplemented with 0.1% sodium pyruvate (TSA-SP). The injury to EHEC O157:H7 cells by hydrogen peroxide stress has been carefully investigated [17]; the authors reported recovering the nonculturable cells by treating them with sodium pyruvate. There was no significant difference between the culturability of F2 plated on TSA-SP and F2 plated on TSP (Fig. 2), but plating the nonculturable EHEC O157:H7 cells of patient strain P5 and the F2 isogenic variants on TSA-SP led to the recovery of culturability for up to 48 h post-incubation (Fig. 2). The BacLight assay showed a 10.5% retention of green fluorescence, even 48 h post-incubation (Table 1), which was also in agreement with the finding that some EHEC O157:H7 cells maintained their viability by acquiring a resuscitation-enabling property. In addition, NaCl-stressed nonculturable cells could be

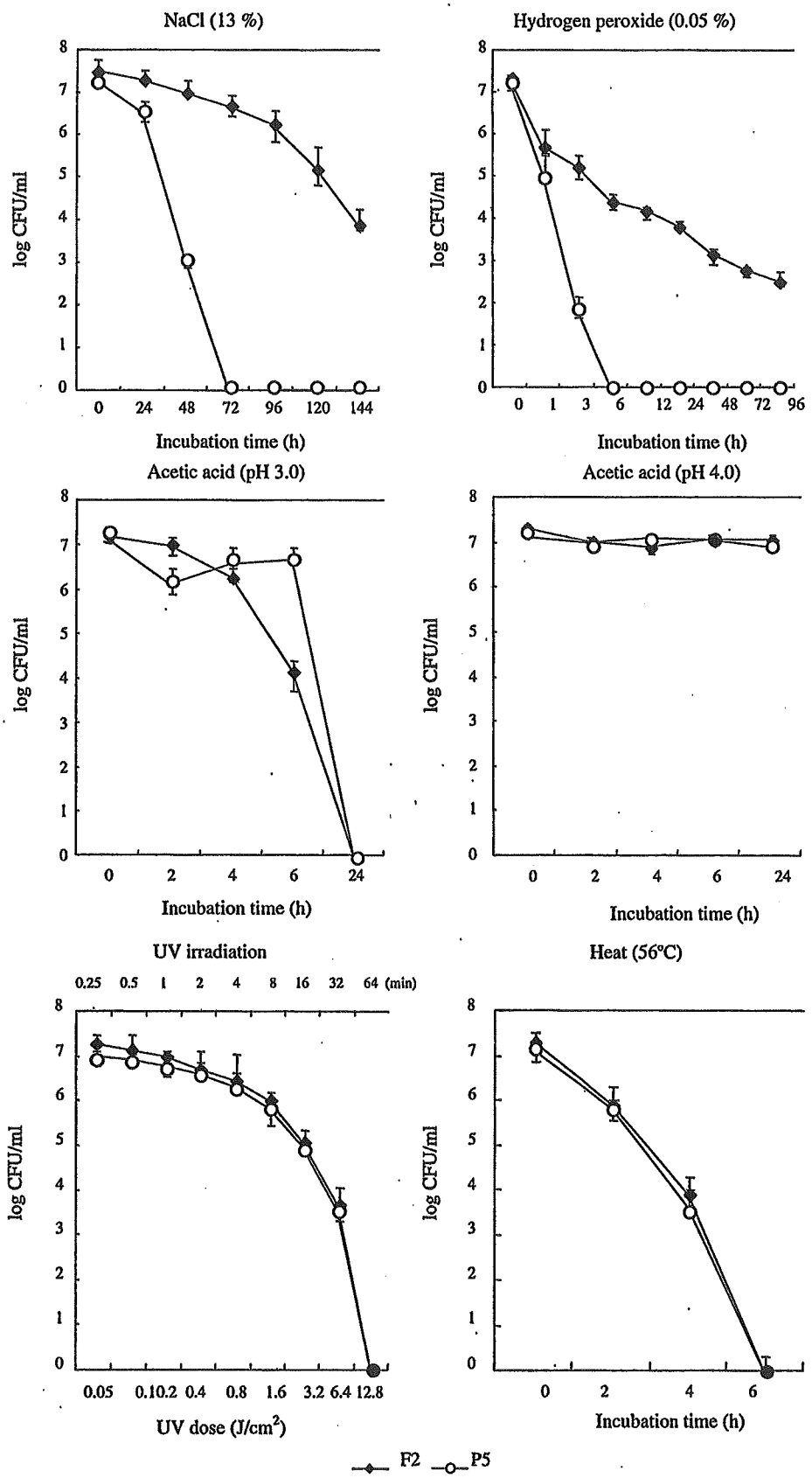

Fig. 1. Culturability of EHEC O157:H7 isolates F1 from food (□) and P5 of patient (○) origin with duration of exposure to environmental stresses. Approximately 10^8 stationary O157:H7 cells were exposed to stress caused by high osmolarity (13% NaCl), oxidative conditions (0.05% hydrogen peroxide), acidic environment (pH 3.0 and 4.0), UV-C radiation (0.05–12.8 J/cm^2 for 15 s to 64 min), or heat (56 °C for 6 h). The culturable EHEC O157:H7 were quantified by plate counts on TSA.

Table 1
Survival and resuscitation properties of *E. coli* O157 cells under oxidative and osmotic stresses

Strain	Incubation time (h)	0.05% Hydrogen peroxide				13% NaCl			
		Plate counts (CFU/ml)			BacLight[a] (%)	Plate counts (CFU/ml)			BacLight (%)
		TSA	TSA-SP	MPN		TSA	TSA-SP	MPN	
F2	0	2.2×10^{7}	1.9×10^{7}	>2400	100.0	4.9×10^{7}	4.7×10^{7}	>2400	100.0
	1	3.4×10^{5}	4.6×10^{5}	>2400	81.8	4.5×10^{7}	4.2×10^{7}	>2400	96.2
	2	1.5×10^{5}	2.1×10^{5}	>2400	74.1	2.8×10^{7}	2.9×10^{7}	>2400	92.2
	3	8.1×10^{4}	7.9×10^{4}	460	56.7	2.1×10^{7}	1.9×10^{7}	>2400	90.9
	6	5.9×10^{3}	8.1×10^{3}	43	51.2	1.8×10^{7}	1.8×10^{7}	>2400	89.4
	12	3.3×10^{3}	3.9×10^{3}	43	24.9	1.6×10^{7}	1.7×10^{7}	>2400	90.1
	24	7.2×10^{2}	6.4×10^{2}	15	12.6	1.3×10^{7}	8.4×10^{6}	>2400	84.9
	48	2.8×10^{2}	3.4×10^{2}	7.3	8.7	8.8×10^{6}	7.9×10^{6}	>2400	84.2
	72	4.3×10^{1}	6.7×10^{1}	ND	3.6	4.3×10^{6}	6.7×10^{6}	>2400	78.0
	96	3.1×10^{1}	4.5×10^{1}	ND	0.9	3.1×10^{6}	4.5×10^{6}	>2400	20.9
	120	4.0×10^{0}	4.0×10^{0}	ND	<0.1	2.8×10^{5}	4.0×10^{5}	>2400	5.1
	144	ND[b]	ND	ND	<0.1	6.9×10^{3}	9.4×10^{3}	460	<0.1
MP37	0	1.5×10^{7}	1.5×10^{7}	>2400	100.0	3.9×10^{7}	3.8×10^{7}	>2400	100.0
	1	6.7×10^{6}	7.2×10^{6}	>2400	77.4	8.9×10^{6}	7.7×10^{6}	>2400	93.7
	2	1.5×10^{3}	2.7×10^{6}	210	74.7	7.7×10^{6}	6.9×10^{6}	>2400	92.0
	3	8.9×10^{1}	1.4×10^{6}	15	50.2	7.1×10^{6}	6.8×10^{6}	>2400	91.1
	6	ND	3.1×10^{4}	ND	48.6	6.8×10^{6}	5.9×10^{6}	>2400	89.5
	12	ND	8.9×10^{3}	ND	29.7	6.1×10^{6}	6.8×10^{6}	1100	90.2
	24	ND	6.4×10^{3}	ND	12.7	3.2×10^{6}	6.4×10^{6}	93	84.6
	48	ND	6.1×10^{0}	ND	10.5	1.8×10^{3}	6.1×10^{3}	15	78.4
	72	ND	ND	ND	1.4	ND	4.0×10^{0}	ND	18.8
	96	ND	ND	ND	<0.1	ND	ND	ND	2.1
	120	ND	ND	ND	<0.1	ND	ND	ND	<0.1
	144	ND	ND	ND	<0.1	ND	ND	ND	<0.1

[a] Percentages of green-fluorescing cells are shown.
[b] ND, not detected.

recovered significantly when grown on TSA-SP (Table 1), indicating that the induction of the VNC by animal passage and the subsequent exposure to foreign stresses might be caused by the same mechanism even when the stresses are different. These data suggested that the decreases in the survival of the patient strain P5 and the F2 isogenic variants were due to the induction of the VNC state, and that in vivo passage caused changes in their susceptibilities to both high osmolarity and oxidative stress.

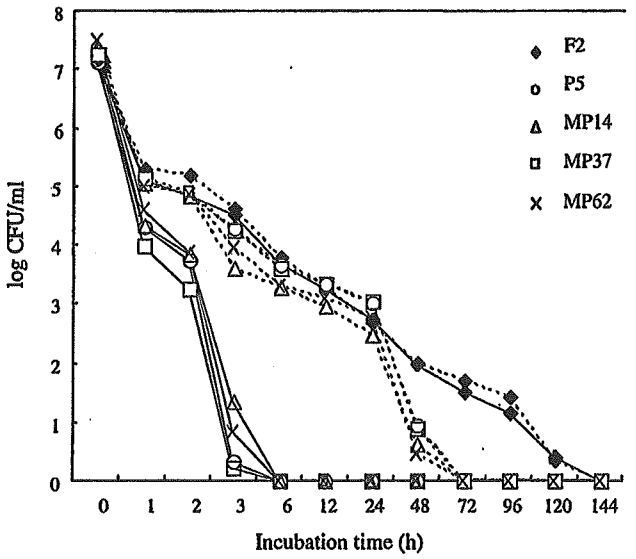

Fig. 2. Culturability and viability in a 0.05% hydrogen peroxide solution of EHEC O157:H7 isolates F1 from food (◆), P5 of patient (O) origin, and three isogenic variants of F2 (MP14, △; MP37, □; MP62, ×) obtained by mouse passage. The numbers of culturable and recoverable cells were quantified by plate counts on TSA (solid lines) and TSA containing 0.1% sodium pyruvate (TSA-SP, dotted lines).

3.4. Reduction in RpoS expression correlates with the culturability of EHEC O157:H7 under high osmolarity and oxidation stress

E. coli have a complex regulatory system that mediates their response to the external environment. A representative system for such regulations is the sigma factors. In particular, one RNA polymerase sigma subunit, RpoS, found in *E. coli* and *Salmonella*, is synthesized and activated to protect the cells from various stresses, and it is well known that NaCl high osmolarity induces the expression of RpoS to aid *E. coli* survival [18]. Since mouse passage caused a subpopulation of EHEC O157 cells to become sensitive to both osmotic and oxidative stresses, we wondered whether oxidative stress would upregulate RpoS expression to the same extent that NaCl did. Although there was no significant difference in RpoS expression levels between strains F2 and MP37 in the stationary phase, strain F2 increased

its RpoS in response to 2 h of oxidative or osmotic stress by 3.74- and 2.41-fold, respectively, compared with the stationary-phase bacteria (Fig. 3). In contrast, the increase in RpoS expression levels in strain MP37 in response to either stress was lower: 1.85-fold for oxidative stress and 1.37-fold for osmotic stress (Fig. 3). The fact that EHEC O157:H7 cells that were easily induced to enter the VNC state in response to osmotic and oxidative stresses had a weaker RpoS expression response, suggested that negative modulations in the mechanisms that protect cells from stress, such as RpoS expression, may occur during in vivo passage, thus causing reduced culturability under these stresses, as appears to be the case with strain MP37.

Although the precise effect of RpoS on the maintenance of viability in the VNC state is still unclear, even strain MP37 expressed RpoS under these stresses (Fig. 3). Bang et al. [19] reported increased RpoS responses in *Salmonella* after phagocytosis. Furthermore, VNC *E. coli* have been isolated from urine specimens of mice and humans [20]. Since phagocytosed bacteria are attacked oxidatively early on, EHEC O157:H7 might quickly enter the VNC state during an infection of mammals, thereby causing underestimation of the viable bacterial numbers using commercial enrichment media.

Overall, in vivo passage negatively influenced RpoS expression in response to environmental stresses, and subsequent exposure to osmotic and oxidative stresses may induce the VNC state in EHEC O157:H7. This bacterial physiological modulation may occur as a compensatory stress response to maintain viability at the partial expense of maintaining RpoS-dependent culturability. Despite the fact that large numbers of EHEC O157:H7 outbreaks occur, there have been only limited cases in which EHEC O157:H7 was detected in the causative foods of the outbreak [21]. The fact that more than one factor induced the VNC state in EHEC O157:H7 in this study suggests the hypothesis that the induction of the VNC state in foods may explain the limited number of cases in which EHEC O157:H7 can be detected. Thus, taking the VNC state of bacteria into consideration in the field of food inspection could help unravel the epidemiological route of infection. Since sodium pyruvate resuscitated EHEC O157:H7 cells from the VNC state they had entered in response to oxidative and osmotic stresses, it might be globally useful for recovering EHEC O157:H7 bacteria from the VNC state. In further studies, we will seek to specify the host factors that cause the phenotypic change, and will investigate the mechanisms of the bacterial adaptive responses in the VNC state, as an approach to establishing a detection and recovery system for pathogenic bacteria in the VNC state.

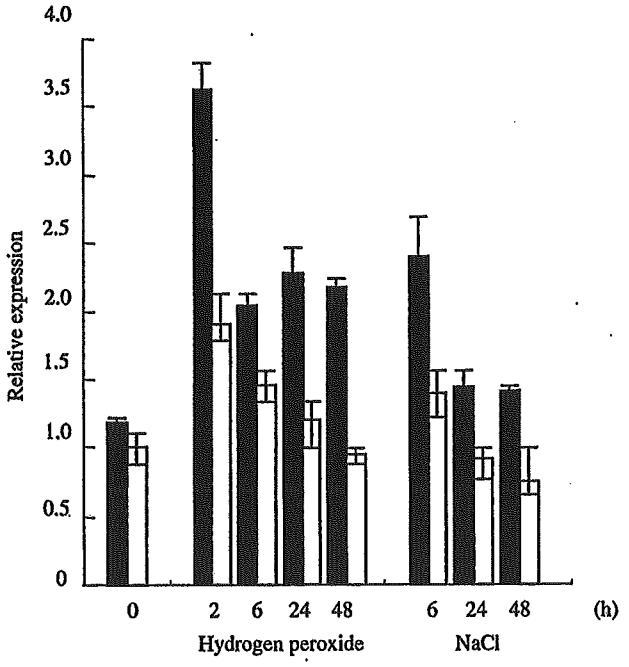

Fig. 3. Expression of RpoS in EHEC O157:H7 strains F2 (closed bars) and MP37 (open bars) under oxidative and osmotic stresses. Western blotting was performed using a rabbit *anti*-RpoS polyclonal antiserum, and the signals were quantified using Quantity One software (Bio-Rad laboratories). Each mean was plotted as a relative value where the expression at 0 h in strain MP37 was defined as 1.0.

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Resuscitation of the viable but non-culturable state of *Salmonella enterica* serovar Oranienburg by recombinant resuscitation-promoting factor derived from *Salmonella* Typhimurium strain LT2

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Abstract

A gene encoding the resuscitation-promoting factor (Rpf) from *Salmonella* Typhimurium LT2 was cloned and characterized. The amino acid sequence encoded by *S.* Typhimurium LT2 *rpf* gene shares 24.2% homology with *Micrococcus luteus* Rpf, which is secreted by growing cells, and required to resuscitate from viable but non-culturable (VNC) state. The *S.* Typhimurium LT2 *rpf* gene is 696 bp long, and shared a conserved segment with *Salmonella enterica* serovar Oranienburg (99.4%). Recombinant Rpf (rRpf) proteins of *S.* Typhimurium LT2 after expression in *E. coli* BL21 harboring the pET15-b plasmid was approximately 25 kDa. Since *S.* Oranienburg cells are relatively quick to enter the VNC state just after incubating in the presence of 7% NaCl at 37 °C for 3 days, we evaluated the biological effect of rRpf by using *S.* Oranienburg VNC cells. The rRpf not only promoted proliferation but also induced resuscitation of VNC cells to the culturable state in a dose-dependent manner. Therefore, rRpf may be useful for detection of bacterial contaminants present in the VNC form in food samples and the environment.

Keywords: *Salmonella* Typhimurium LT2; *Salmonella enterica* serovar Oranienburg; Viable but non-culturable state; Resuscitation-promoting factor

1. Introduction

Several food poisoning bacteria are known to enter into a viable but non-culturable (VNC) state in response to adverse environmental conditions, in which they do not longer grow on conventional media, but remain high viability and retain pathogenicity (Roth et al., 1988; Nilsson et al., 1991; Rahman et al., 1994; Stern et al., 1994; Colwell et al., 1996; Pommepuy et al., 1996; Ravel et al., 1995; Whitesides and Oliver, 1997). For example, *Vibrio cholera, Escherichia coli* O157:H7, *Campylobacter jejuni* and *Salmonella* spp. enter into the VNC state in environments such as seawater, ground water, rivers and salted food (Colwell et al., 1985; Rollins and Colwell, 1986; Barcina et al., 1997; Choa and Kim, 1999; Makino et al., 2000; Mattick et al., 2000; Santo Domingo et al., 2000). From a food hygiene perspective, it often becomes a problem that contamination of such VNC cells in food may be overlooked under standard routine laboratory procedure, resulting in improper assessment of contaminated pathogens in food and environment.

In 1999, an outbreak across 46 prefectures of Japan, involving septicemia and gastroenteritis, resulted after consumption of dried processed squid contaminated with *Salmonella enterica* serovar Oranienburg (Tsuji and Hamada, 1999; Saito et al., 2000). Since *S.* Oranienburg was detected in the environment of the processing plant as well as in waste floating around the import port, the raw squid was probably contaminated during the shipping and production process. In general, the preservation of food by salt followed by drying has been used widely as an effective means of preventing food-borne infections. We have found that *Salmonella* Oranineburg becomes non-culturable under osmotic stress, but retains its pathogenicity (Asakura et al., 2002).

Although a large body of evidence has been accumulated, little is known about genetic pathways underlying the VNC

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state. A number of in vitro methods have been evaluated for resuscitation of non-culturable bacteria. Resuscitation-promoting factor (Rpf) is a small protein, originally identified in *Micrococcus luteus*, which promotes recovery of bacteria from a VNC state to a vegetative state (Kaprelyants et al., 1996; Mukamolova et al., 1998). Purified *M. luteus* Rpf can stimulate growth and increase the recovery of *M. luteus* bacteria as well as *Mycobacterium tuberculosis* from prolonged stationary growth cultures. Genes encoding Rpf-like proteins are widely distributed throughout the pathogenic bacteria such as mycobacteria, corynebacteria, and streptomycetes (Mukamolova et al., 1998; Kell and Young, 2000; Hartmann et al., 2004).

Because VNC *S.* Oranienburg cells can resuscitate and remain pathogenic under favorable conditions (Asakura et al., 2002), which make them potential threats to the human health, it is of major importance to develop a new procedure for detection and isolation of them from food and environment samples. To this end, we surveyed resuscitation-related factors in the *Salmonella* genome database, and found that *Salmonella* Typhimurium strain LT2 has an *rpf*-like gene with 24.2% amino acid homology to *M. luteus* Rpf. We cloned and characterized this *rpf*-like gene using a polymerase chain reaction (PCR) method, and evaluated its resuscitation effect on the VNC state of *S.* Oranienburg which has high homology of nucleic acid and amino acid sequences to *S.* Typhimurium LT2 and is easy to induce VNC state. The recombinant protein of *rpf* gene resuscitated the VNC cells of *S.* Oranienburg that were induced by osmotic stress, and its effect was dose-dependent. These results have significant implication for detection of microbial contamination in food.

2. Materials and methods

2.1. Bacterial strains and media

Bacterial strains used in this study are shown in Table 1. *S.* Typhimurium strain LT2 was used for recombinant Rpf (rRpf) protein production. *S.* Oranienburg Sa99004, isolated from a patient in an outbreak caused by dried processed squid, was used to produce VNC state (Asakura et al., 2002). *E. coli* DH5 α and BL21 (λDH3) were used as the non-expression host and the expression host, respectively. The liquid culture media included nutrient broth (NB; Difco Lab., MI, USA) lacking NaCl, brain heart infusion broth (BHI; Difco Lab.), trypticase soy broth (TSB; BBL/Becton Dickinson, MI, USA), and Luria broth (LB; BIO 101, Inc., CA, USA) with/without 50 μg/ml ampicillin. Agar plates were made from desoxycholate hydrogen sulfide lactose agar (DHL; Eiken, Tokyo, Japan), trypticase soy agar (TSA), and LB by adding 1.5% agar with/without 50 μg/ml ampicillin.

Table 1
Bacterial strains used in this study

Bacterial strain	Description or genotype	References
Salmonella Typhimurium LT2	rRpf production	Laboratory stock
Salmonella Oranienburg Sa99004	Salt sensitive strain, human isolate	Asakura et al., 2002
E. coli DH5α	Non-expression host	Laboratory stock
E. coli BL21 (λDH3)	Expression host	Laboratory stock
TP	*E. coli* TOP10F'-pCR2.1TOPO::*rpf*	This study
DP	*E. coli* DH5 α-pET15b::rpf	This study
BP	*E. coli* BL21 (λDH3)-pET15b::rpf	This study

2.2. Cloning of S. Typhimurium LT2 and S. Oranienburg rpf

The complete sequence of *S. typhimirium* LT2 genome was determined (McClelland et al., 2001, Genbank accession number: NC_003197). By a BLAST search of the similar sequence to M. luteus *rpf* in the GenBank database, we have found that *S.* Typhimurium LT2 has a gene with 24.2% homology. Based on this data, primers were designed to amplify the *rpf*-like gene to be used in PCR-based cloning for production of the rRpf protein. The upstream primer was 5′-CCCATATGCGAATTCTGGCTATCGATACC-3′, and the downstream primer was 5′-CCGGATCCTCATTCTTTGCC-GGGAAGTTTCTT-3′. The reaction mixtures were subjected to 1 cycle of 4 min at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 1 cycle of 7 min at 72 °C. Ten reaction mixtures were pooled, and the PCR product was purified with QIAquick PCR purification kit (QIAGEN, MD, USA) according to the supplier's protocol and inserted into the TA TOPO cloning vector (Invitrogen, CA, USA). After successful cloning, the plasmid was extracted by QIA plasmid miniprep (QIAGEN) and the presence and orientation of the insert were determined by restriction analysis using restriction endonucleases *Bam*HI and *Nde*I (Takara, Shiga, Japan). The inserted sequences were analyzed by ABI PRISMS 310 Genetic Analyzer (Applied Biosystems, CA, USA) with M13 Forward and M13 Reverse primers. The *Bam*HI and *Nde*I fragments were cloned into the pET-15b expression vector (Novagen, Darmstadt, Germany) and transformed into *E. coli* DH5 α (DP). After plasmid extraction and determination of the insert by restriction enzyme digestion, the plasmid was transformed into *E. coli* BL21 (BP) for expression of the protein.

2.3. Expression and purification of histidine-tagged recombinant Rpf protein

The BP were grown overnight in LB media supplemented with 50 μg/ml ampicillin at 37 °C. Bacterial suspension (1 ml) was added to 50 ml fresh LB supplemented with ampicillin (50 μg/ml) and incubated at 37 °C to an optical density value at 600 nm (OD600) of 0.6. Gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Wako, Tokyo, Japan) for 4 h; during induction, cultures were shaken at 37 °C. The bacterial cultures were pelleted and processed for histidine-tagged protein purification according to the supplier's protocol (Ni–NTA agarose, QIAGEN). After purification, the presence of the histidine-tagged protein was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and western blot with an anti-histidine-tagged Ab (Amersham Biosciences KK, Tokyo, Japan).

2.4. Determination of purified protein by SDS-PAGE and western blot

SDS-PAGE was performed using a 12% polyacrylamide separating gel and a 5% stacking gel. Aliquots (80 μl) of extracted rRpf protein were suspended in 20 μl of 5× sample buffer, heated for 5 min at 100 °C, and immediately chilled on ice. The samples were separated in an electrophoretic cell (Mini Protean 3; Bio-Rad, CA, USA) at a constant current (20 mA per gel) for 1 h.

The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) in an electrophoretic semi-dry transfer cell (Trans-Blot SD; Bio-Rad) at 15 V for 1 h. After transfer was completed, the PVDF membranes were incubated overnight in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 5% skim milk to block nonspecific binding sites and washed three times with PBS-T. The membranes were then incubated at room temperature for 1 h with histidine-tagged (Ag-1) monoclonal Ab (clone 13/45/31/2; Oncogene, Biosciences Inc, CA, USA) diluted 1:100 in PBS-T containing 0.5% skim milk and washed as described above. Thereafter, the membrane were incubated at room temperature for 1 h with peroxidase-conjugated $F(ab')_2$ fragment sheep anti-mouse IgG (Amersham Biosciences) diluted 1:5000 in PBS-T containing 0.5% skim milk. The blots were washed in PBS-T and bound peroxidase enzyme was detected with the ECL-Plus western blotting detection reagent (Amersham Biosciences).

2.5. Antibody production and purification

To obtain anti-rRpf polyclonal Ab, we immunized Japanese White rabbits (Charles River Japan, Tokyo, Japan) four times at 2-week intervals by subcutaneous injection with 1 ml of a 50% (v/v) mixture of rRpf (2 mg/ml in PBS) and Freund's adjuvant (Sigma, MO, USA). Complete Freund's adjuvant was used for the first immunization and incomplete Freund's adjuvant for the subsequent immunization. Whole blood was collected at 2 days after the last immunization, and IgG was purified from serum by using a protein G-conjugated column (MAbTrap™ kit, Amersham Biosciences).

2.6. Induction of S. Oranienburg VNC state

S. Oranienburg cells grown at 37 °C for 20 h in NB were washed three times with sterile normal saline. Approximately, 10^9–10^{10} cells were suspended in 100 ml of 7% NaCl solution and incubated at 37 °C for 3–4 days. Everyday, a 0.1 ml aliquot of the suspension and its serial dilutions were spread on DHL and TSA plates to measure the CFU. To ensure the data were accurate, the experiment was repeated in duplicate.

2.7. Determination of viability

S. Oranienburg cells in 7% NaCl solution were stained by using the Live/Dead BacLight Bacterial Viability kit (Molecular Probes, OR, USA). The stained bacteria were examined under fluorescence microscopy (Olympus BX51, OPELCO, VA, USA) and the viability of 1000 cells was assessed.

2.8. Resuscitation of VNC cells by purified rRpf protein

Bacterial cells in 7% NaCl solution (1 ml) which entered the VNC state were inoculated in 10 ml of 5% BHI containing 10 μg/ml of purified rRpf, followed by incubation for 7 days at 30 °C. To examine the effect of purified rRpf on resuscitation, 5% BHI without purified rRpf was used as negative control and 5% BHI enriched with 100 μl of filter-sterilized NB culture supernatant from an overnight culture of *S.* Oranienburg Sa99004 strain served as positive control (Asakura et al., 2002). To determine the dose-dependency of rRpf protein, various concentrations of protein (0, 0.001, 0.01, 0.1, 1 and 10 μg/ml) were added to 5% BHI, followed by the VNC cells (1 ml) and incubated at 30 °C for 2–3 days. The colony forming units was assessed on TSA to examine the dose-dependent effect.

2.9. Inhibitory effect of anti-rRpf Ab on resuscitation

S. Oranienburg VNC cells (1 ml) were inoculated into 10 ml of 5% BHI containing rRpf at 0, 1, 2, 5 or 10 μg/ml. Then, rabbit anti-rRpf Ab (0, 1, 2, 5 or 10 μg/ml) was added and resuscitation was monitored by bacterial growth and colony count on TSA during 7 days incubation at 30 °C.

2.10. Growth enhancement by rRpf protein

Approximately, 10^2–10^3 or $>10^5$ cells of overnight culture of *S.* Oranienburg, which were washed twice with sterile PBS by centrifugation at 3500 rpm for 10 min at 4 °C were added to 10 ml of TSB, which supplement with rRpf protein at concentrations 0, 1, 5 and 10 μg/ml. After incubation at 37 °C for 0, 2, 4, 6, 8, 10, 12, 14, 16 and 24 h, the OD600 of culture was measured to determine the enhancement ability of the rRpf protein.

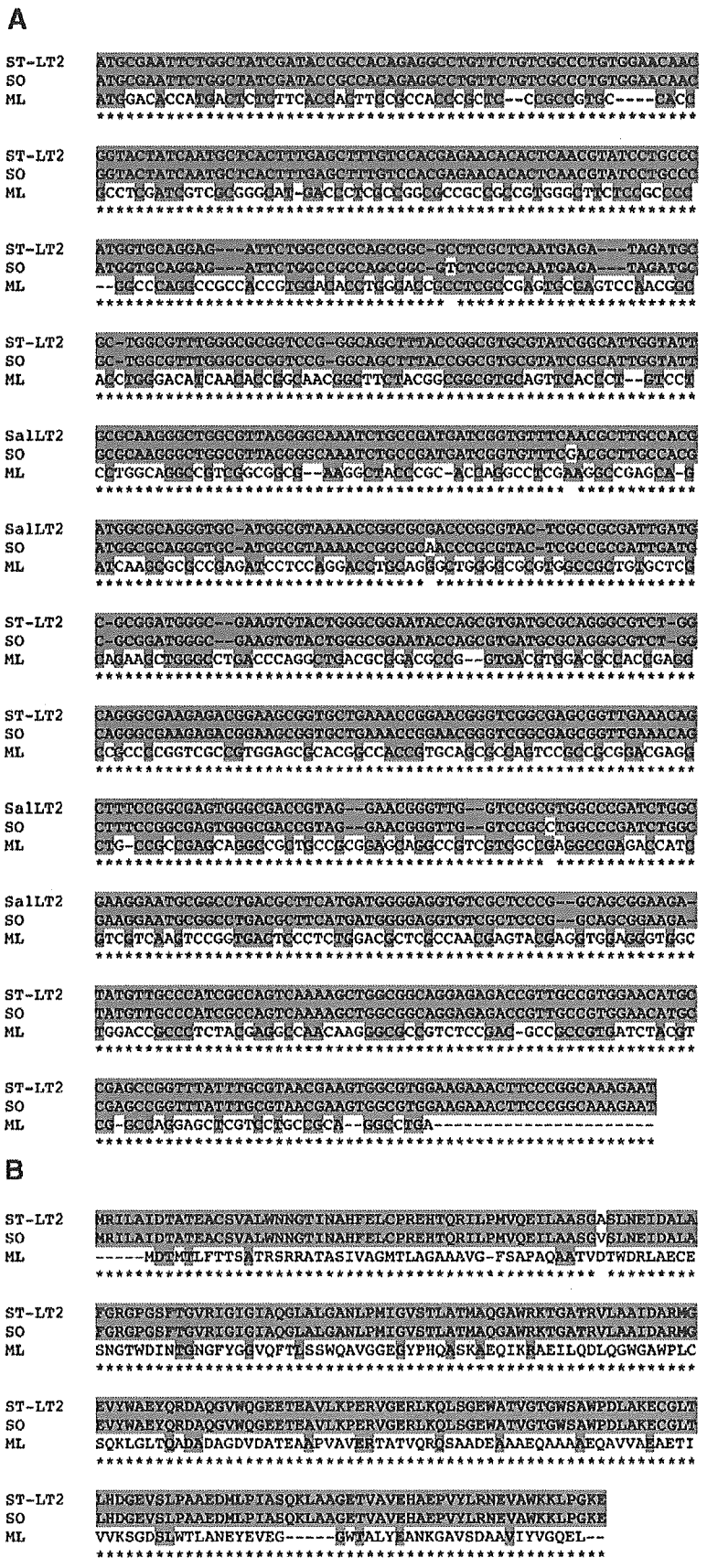
2.11. Nucleotide sequence accession number

The complete genome sequence of *S.* Typhimurium strain LT2 were accessed at GenBank databases as NC003197 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=16763390). The *rpf* gene sequence data of *S.* Typhimurium strain LT2 and *S.* Oranienburg have been submitted to the GenBank databases under accession number AY772265 and AY772266, respectively.

3. Results

3.1. S. Typhimurium LT2 rRpf protein production

A GenBank database search reveals that *S.* Typhimurium LT2 possessed a gene that shared significant nucleotide and amino acid similarity (44.8% and 24.2%, respectively) with the


A
ST-LT2
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ML
SalLT2
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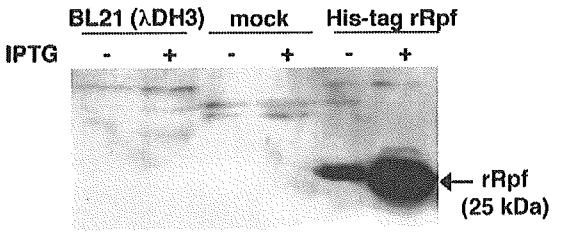

Fig. 2. Western blot analysis of rRpf histidine-tagged protein with anti-histidine-tagged Ab. The rRpf histidine-tagged protein (MW ~25 kDa) revealed only in BP, not in host cell or mock control. (−); before induction, (+); after 4 h induction with 1 mM IPTG.

M. luteus rpf gene (Fig. 1). Based on the sequence of this gene, primers were designed for PCR-based molecular cloning of the *S.* Typhimurium LT2 *rpf*-like gene, and determined the sequence of the PCR products. The *S.* Typhimurium *rpf*-like gene was 696 bp and has 99.4% nucleotide and 99.6% amino acid similarity with the homologous gene in *S.* Oranienburg (Fig. 1).

To determine the biological role of *rpf*-like gene, we produced rRpf in *E. coli* expression system. Immunoblot analysis with anti-histidine-tagged Ab reveals that the molecular weight of rRpf was 25 kDa (Fig. 2). The rRpf protein was found only in BP, not in the host *E. coli* BL21 or the mock control (Fig. 2). This protein was stable after heat treatment at 55 °C for 15 min as determined by its resuscitation ability. However, its activity was lost when heated at 100 °C for 15 min (data not shown).

3.2. Induction of S. Oranienburg VNC cells by osmotic stress

When 10^7–10^8 cells/ml of *S.* Oranienburg in the stationary phase were cultured in 7% NaCl at 37 °C, the culturable and viable cells were measured. The cultivability of the bacterial suspension was determined in each condition, tested by spread plate counting, and the cell viability was determined by the propidium iodide and SytoxGreen double staining. Before incubation in 7% NaCl, the colony forming units (CFU/ml) of *S.* Oranienburg were approximately 1.45×10^9. The culturability of *S.* Oranienburg drastically dropped to 1.57×10^5 and 1.42×10^3 CFU/ml on days 1 and 2 post-incubation, respectively, and was completely lost after 3 days incubation (Fig. 3). Although, the CFU of *S.* Oranienburg dramatically decreased, the viability only slowly declined and remained high after 3 days post-incubation, when no colonies appeared on TSA. The viability of *S.* Oranienburg before incubation in 7% NaCl was approximately 92%. The viable cells slightly decreased and remained as high as 84%, 80% and 76% at days 1, 2 and 3 post-incubation, respectively (Fig. 3). Thus, we concluded that 7% NaCl forced *S.* Oranienburg to enter a VNC state by 3 days incubation.

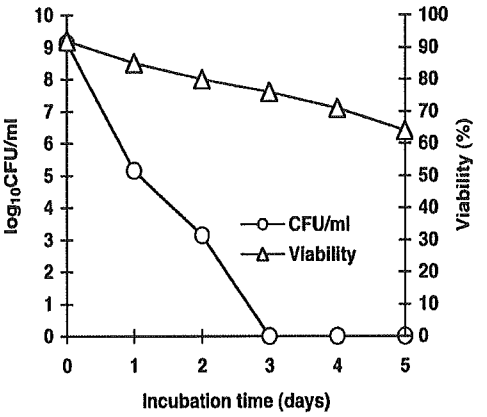

Fig. 3. Growth and viability of *S.* Oranienburg during incubation in 7% NaCl. The growth was determined by colony forming units of bacteria on TSA agar after overnight incubation at 37 °C (○). Fluorescence staining by Live/Dead BacLight kit was used to assess the viability of the bacteria (△).

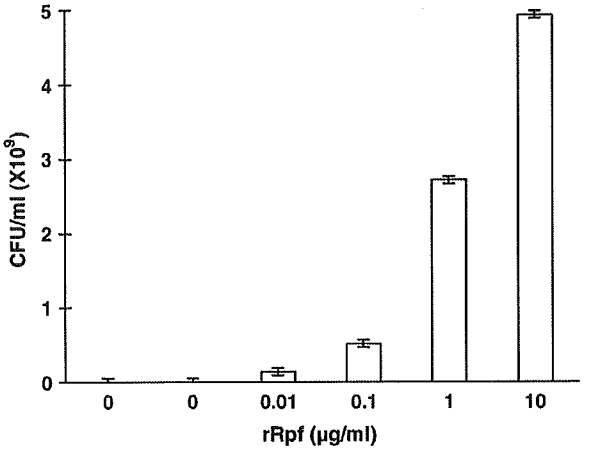

Fig. 4. Dose-dependency of rRpf protein. One milliliter of the VNC cells of *S.* Oranienburg was added to 5% BHI supplemented with 0, 0.001, 0.01, 0.1, 1 or 10 μg/ml of rRpf protein, followed by incubation at 30 °C. After 2 days of incubation, the colony forming units was determined by plating on TSA agar.

3.3. Resuscitation of the VNC state by rRpf protein and dose-dependency

Next, the resuscitation activity of rRpf was examined. The *S.* Oranienburg VNC cells were incubated at 30 °C for 7 days with various concentrations (0, 0.001, 0.01, 0.1, 1 and 10 μg/ml) of rRpf in BHI medium. While no colonies were formed on TSA plates when rRpf was not added, 1.42×10^8, 5.18×10^8, 1.63×10^9, 2.72×10^9 and 4.94×10^9 CFU/ml of *S.* Oranienburg were recovered by the addition of rRpf at 0.01, 0.1, 1 and 10 μg/ml, respectively. However, 1 ng/ml of rRpf no longer had any effect on resuscitation (Fig. 4). The rRpf protein resuscitated *S.*

Fig. 1. Alignment of nucleotide and amino acid encoded by rpf gene of *S.* Typhimurium LT2 (ST-LT2), *S.* Oranienburg (SO) and *M. luteus* (ML). (A) Nucleotide alignment of ST-LT2, SO and ML *rpf*. (B) Amino acid alignment of Rpf from ST-LT2, SO and ML. The sequences were aligned using the ClustalW multiple alignment algorithm from DNA Data Bank of Japan (DDBJ). The homologous portions are boxed with dark background and non-homologies have white background. The homologies between ST-LT2 and SO are indicated by (*).

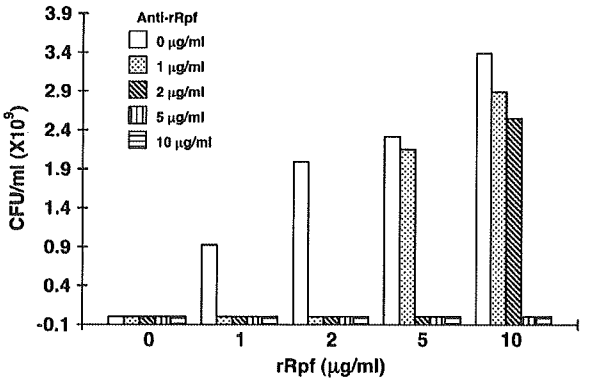

Fig. 5. Inhibitory effect of anti-rRpf Ab on rRpf-dependent resuscitation. Anti-rRpf Ab (0, 1, 2, 5 or 10 μg/ml) were added to 5% BHI containing rRpf (0, 1, 2, 5 or 10 μg/ml), which was inoculated by VNC cells of *S.* Oranienburg. During 7 days incubation at 30 °C, the resuscitation inhibitory effect was determined by bacterial growth and plating on TSA.

Oranienburg from VNC state to culturable state in a dose-dependent manner.

3.4. Inhibitory effect of anti-rRpf Ab on rRpf-dependent resuscitation

VNC cells of *S.* Oranienburg in 7% NaCl (1 ml) were inoculated into 10 ml of 5% BHI supplemented with rRpf at 0, 1, 2, 5 or 10 μg/ml in the presence or absence of rabbit anti-rRpf Ab (0, 1, 2, 5 or 10 μg/ml). The resuscitation was monitored by bacterial growth and colony count on TSA during 7 days incubation at 30 °C. The addition of rRpf to the culture recovered *S.* Oranienburg from the VNC state to the culturable state. However, the addition of various concentrations of anti-rRpf Ab together with rRpf abolished the resuscitation process induced by rRpf (Fig. 5). To inhibit rRpf-dependent resuscitation completely, more than half concentrations of Ab added to rRpf were required.

3.5. Growth enhancement by rRpf protein

We also examined whether rRpf has a proliferation stimulating activity to *S.* Oranienburg. The results showed that rRpf could enhance the growth of *S.* Oranienburg cells in a dose-dependent manner (Fig. 6A). However, when the inoculum was increased to $>10^5$ cells, the rRpf did not show any significant growth enhancement (Fig. 6B).

4. Discussion

Since its first description in 1985, the VNC response has been observed in a number of bacteria (Colwell et al., 1985; Rollins and Colwell, 1986; Roth et al., 1988; Nilsson et al., 1991; Rahman et al., 1994; Stern et al., 1994; Colwell et al., 1996; Pommepuy et al., 1996; Ravel et al., 1995; Barcina et al., 1997; Whitesides and Oliver, 1997; Choa and Kim, 1999; Makino et al., 2000; Mattick et al., 2000; Santo Domingo et al., 2000). Food-borne bacteria in a VNC state have been of concern as a significant problem for food hygiene and public health. Although PCR has been widely applied to detect bacterial contamination in food, it only can tell us the presence or absence of bacterial DNA, not the state such as dead or dormant. So far, there are no simple methods for detection or recovery of VNC cells.

We previously demonstrated that *S.* Oranienburg enters the VNC state following the exposure of osmotic stress, and the addition of mid-log phase *S.* Oranienburg culture supernatants can resuscitate the dormant cells (Asakura et al., 2002), suggesting that viable bacteria secret a resuscitation-related molecule in a soluble form. In this study, we identified the gene encoding Rpf in *S.* Typhimurium LT2 and *S.* Oranienburg, and demonstrated that rRpf was able to resuscitate *S.* Oranienburg cells from VNC state in dose-dependent manner. We also have found a *rpf*-like gene in *E. coli* O157:H7, which has 76% homology in amino acid sequence with *S.* Typhimurium LT2 *rpf*. However, *S.* Typhimurium LT2 rRpf protein showed no effect on resuscitation of *E. coli* O157:H7 VNC cells (data not shown), suggesting that it may specific to *S.* Oranienburg or other *Salmonella* spp., which share high homology in *rpf* gene sequence.

Mukamolova et al. (1998) have shown that purified *M. luteus* Rpf can stimulate growth and increase recovery of *M. luteus* bacteria as well as *Mycobacterium tuberculosis* bacteria from prolonged stationary cultures. We evaluated the growth stimulation effect by our rRpf. The rRpf could slightly enhance

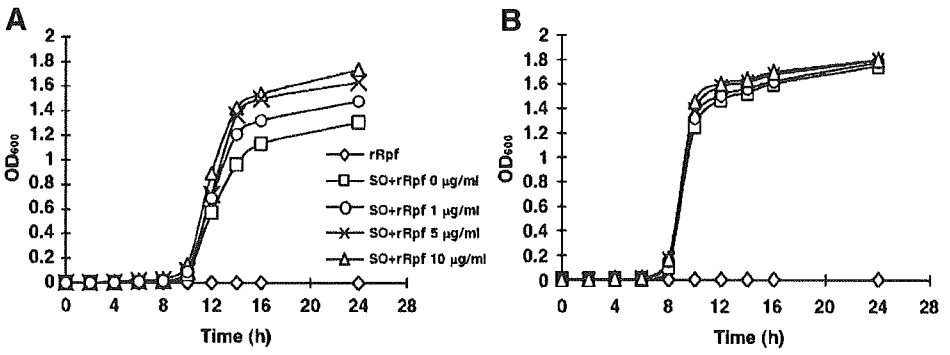

Fig. 6. Growth enhancement by rRpf protein. The overnight culture of *S.* Oranienburg (SO) was added to TSB containing rRpf protein at 0, 1, 5 or 10 μg/ml and incubated at 37 °C with shaking. OD_{600} was measured at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 24 h after incubation. (A) Growth enhancement effect of rRpf when approximately 200 cells were inoculated to TSB. (B) Enhancement effect when $>10^5$ cells were used.

the growth of viable and culturable *S.* Oranienburg at low cell densities, but not at a high-density population. In addition to its ability in resuscitation of non-culturable cells, Rpf may work as a bacterial growth factor dependent on population density. However, the growth stimulation by Rpf appears to be only a minor effect. The molecular mechanism of how Rpf resuscitates dormant non-culturable cells is unknown. In our study, the addition of rabbit anti-Rpf Ab inhibited resuscitation effect by Rpf, suggesting the binding of Ab to Rpf block the ligand–receptor interaction that triggers signaling of resuscitation process in VNC cells. Identification of the receptor for Rpf, the profiling the gene expression induced by Rpf stimulation, or analysis of *rpf*-null mutants, would help us to understand the underling mechanism for VNC and resuscitation processes in bacteria.

Bacterial pathogens can contaminate foods at any point during production, processing, transport, retailing, storage or meal preparation. During these processes, some food-borne pathogens can survive in adverse environment by entering VNC status without losing their pathogenicity. The fact that *S.* Oranienburg colonies were detected and increased in number by the incubation with Rpf implies an underestimation of the real amount of active population by regular culturing methods. It will bring up a greater threat to microbial food safety and will need additional efforts in the development of new food inspection techniques. Our results suggest that it may be possible that rRpf can be used for the recovery and detection of VNC bacteria contaminated in food samples and the environment. For example, addition of rRpf to regular culture media may substantially improve laboratory methods for the detection, isolation and cultivation of VNC pathogens. To confirm this, future works need to be performed. It can be applied as a simple, easy, non-special equipped method, and may decrease misdiagnosis of food inspection, and reduce the public risk to *S.* Oranienburg infection.

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