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The Viable But Nonculturable State of Kanagawa Positive and Negative Strains of *Vibrio parahaemolyticus*

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Ingestion of shellfish-associated *Vibrio parahaemolyticus* is the primary cause of potentially severe gastroenteritis in many countries. However, only Kanagawa phenomenon (hemolysin) positive (KP$^+$) strains of *V. parahaemolyticus* are isolated from patients, whereas >99% of strains isolated from the environment do not produce this hemolysin (i.e. are KP$^-$). The reasons for these differences are not known. Following a temperature downshift, *Vibrio parahaemolyticus* enters the viable but nonculturable (VBNC) state wherein cells maintain viability but cannot be cultured on routine microbiological media. We speculated that KP$^+$ and KP$^-$ strains may respond differently to the temperature and salinity conditions of seawater by entering into this state which might account for the low numbers of culturable KP$^+$ strains isolated from estuarine waters. The response of eleven KP$^+$ and KP$^-$ strains of *V. parahaemolyticus* following exposure to a nutrient and temperature downshift in different salinities, similar to conditions encountered in their environment, was examined. The strains included those from which the KP$^+$ genes had been selectively removed or added. Our results indicated that the ability to produce hemolysin did not affect entrance into the VBNC state. Further, VBNC cells of both biotypes could be restored to the culturable state following an overnight temperature upshift.

Key words: *Vibrio parahaemolyticus,* Kanagawa hemolysin, VBNC, resuscitation, survival

Vibrio parahaemolyticus is a ubiquitous Gram negative bacterium which is a normal inhabitant of coastal waters, but rarely reported from open ocean waters. Ingestion of this organism in raw or undercooked shellfish results in gastroenteritis with characteristic symptoms such as vomiting, diarrhea, chills, abdominal cramps, and fever. Symptoms are usually self-limiting within one week or less, with no need for antibiotic treatment (Oliver and Kaper, 2001). Two distinct biotypes of *V. parahaemolyticus* exist. Kanagawa phenomenon positive (KP$^+$) strains are hemolytic, while Kanagawa phenomenon negative (KP$^-$) strains are not. This activity can be observed on Wagatsuma agar, made with freshly-drawn and washed human red blood cells (Watatsuma, 1968). The hemolysin produced is a thermostable direct hemolysin (TDH) encoded by two *tdh* gene copies, *tdh*1 and *tdh*2. The products of these genes are immunologically identical, but differ in seven amino acid residues. The *tdh*2 product, however, is responsible for >90% of the total hemolysin production in KP$^+$ cells (Nishibuchi and Kaper, 1995; Nishibuchi *et al.*, 1992). Significantly, most KP$^-$ strains do not possess the *tdh* genes. Also of importance is the fact that 95% of strains isolated from patients are of the KP$^+$ type, while the great majority of environmental isolates are of the KP$^-$ type (Sakazaki *et al.*, 1968; Miyamoto *et al.*, 1969).

Interestingly, this organism has been shown to demonstrate seasonal variation, more often cultured during the warm summer months as opposed to the cooler winter months (Kaneko and Colwell, 1973; Colwell, 1974; Hackney *et al.*, 1980; Kumazawa and Kato, 1985; Chowdhury *et al.*, 1990). Further, 94% of infections of *V. parahaemolyticus* from 1973 to 1998 in the United States occurred between the months of April and October (Daniels *et al.*, 2000). As suggested by studies reported by Jiang and Chai (1996) and Mizunoe *et al.* (2000), such a seasonal difference may be due to the finding that *V. parahaemolyticus* enters into a viable but nonculturable (VBNC) state upon temperature and nutrient downshift conditions in the laboratory. As the cells enter this state, plate counts decline until this organism can no longer be cultured by routine microbiological methods. However, a significant population of these nonculturable organisms can be shown to retain viability as indicated by a variety of direct microscopic assays (Oliver, 1993).

At least 30 bacterial genera have been shown to enter into this physiological state during such unfavorable conditions as temperature up- or down-shifts, osmotic variations or decreased oxygen levels. These organisms

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include *Escherichia coli, Pseudomonas fluorescens, Salmonella typhimurium*, and *Vibrio vulnificus*, with the latter being the most extensively studied (Oliver, 1993, 2000a). In general, VBNC cells are characterized by a reduction in cell size and decreased DNA, RNA and protein synthesis (Oliver, 1993, 2000b). In addition, the lipid profiles of VBNC cells are altered, probably to maintain membrane fluidity during temperature downshift (Linder and Oliver, 1989; Day and Oliver, 1998).

In order for this state to be recognized as a physiological survival mechanism, VBNC cells must be able to exit or "resuscitate" from this state so that the cells can again be detected on routine microbiological media. Indeed, such resuscitation has been demonstrated in several species and can result from a simple reversal of the conditions that induced the cells to enter the VBNC state, or require such complex factors as the presence and interaction of a protozoan species (Oliver, 1993, 2000a; Steinart *et al.*, 1997; Whitesides and Oliver, 1997). Importantly, *in situ* entry into and exit from the VBNC state has been observed in *V. vulnificus* when nonculturable populations of this organism are placed into an estuarine environment (Oliver *et al.*, 1995).

A study reported by Jiang and Chai (1996) suggested that the difference in culturability of KP^+ and KP^- cells from the environment might be due to variations in the rate they entered the VBNC state. Because that study employed only a single strain of each biotype, the present study was designed to examine what differences, if any, exist between the ability of six KP^+ and five KP^- strains to enter the VBNC state. These included isogenic mutants of a KP^+ strain which had the *tdh1* and *tdh2* genes deleted or restored on a plasmid. Because *V. parahaemolyticus* cells undergo osmotic fluctuations between various seawater habitats, estuarine waters and the human body, osmotic concentrations were varied to determine if they played any role in the time required for entrance into the VBNC state. Known inducers of resuscitation/cultivation were also tested to determine if exit from this dormant state could be demonstrated.

Materials and Methods

Bacterial strains and maintenance of cultures

Eleven *V. parahaemolyticus* isolates, of both clinical and environmental origin, were used in this study (Table 1). Charles Kaysner of the Food and Drug Administration provided all bacterial cultures, except for strain AQ3815 and two derived mutant strains, which were provided by James Kaper of the Center for Vaccine Development of the University of Maryland. Cultures were maintained on heart infusion agar (HI; Difco, USA).

Microcosm preparation

Cells were grown overnight at room temperature to stationary phase in HI broth employing artificial seawater (ASW; Wolf and Oliver, 1992) as the diluent (HIA). Cells from this overnight culture were placed into fresh HIA to provide a final 1% inoculum and grown to log phase (OD 0.15-0.20 at 610 nm) at room temperature with aeration. Cells of this culture were then placed into a microcosm diluent of ASW or 20% ASW (salinity similar to estuarine waters) to provide a final concentration of *ca.* 10^5-10^6 colony forming units (CFU) per ml. Microcosms were incubated at 5°C to induce entry into the VBNC state. Experiments were performed in duplicate in all cases.

Table 1. *V. parahaemolyticus* strains employed in this study

Strain	Kanagawa Phenomenon	Source
SAK11	KP^+	clinical
F11-3A	KP^+	clam
NY477	KP^+	oyster
SPRC851	KP^+	clinical
AQ4037	KP^-	clinical
VP99	KP^-	water
WRI	KP^-	water
1904653	KP^-	shrimp
AQ3815[1]	KP^+	clinical
AQ3815Δ*tdh1*Δ*tdh2*[1]	KP^-	lab modified
AQ3815Δ*tdh1*Δ*tdh2* (pKTN115)[2]	KP^+	lab modified

[1]AQ3815 described in Nishibuchi and Kaper (1990).
[2]Construction of co-isogenic derivatives of AQ3815 described in Nishibuchi *et al.* (1992)

Culturability of cells

Samples from each microcosm were periodically removed, serially diluted in ASW or 20% ASW, and plated onto HI agar. Cells were considered to be nonculturable when less than 0.1 CFU/ml were detected. This limit of detection was obtained by filtering 10 ml of the microcosm onto a 0.2 μm polycarbonate filter (Poretics, USA) and then placing the filter onto the appropriate medium.

Viability assay

Once cells were determined to be nonculturable, microcosm samples were removed and assayed for viability using the substrate responsiveness method of Kogure *et al.* (1979). Briefly, cells were incubated in the dark with 0.025% yeast extract (Difco, USA) as a nutrient source and 0.002% nalidixic acid (Sigma, USA) to inhibit septation of the growing cells. After overnight incubation, samples (1 ml) were fixed with 5% filter-sterilized formalin and stained with 0.1% acridine orange. Samples were then filtered onto a 0.2 μm black polycarbonate filter (Poretics, USA). Epifluorescent microscopy (Olympus model BX60F5) using appropriate filter sets was used to determine viable cell counts (cells which were a minimum of 2x the length of untreated cells).

***In vitro* resuscitation**
After cells entered the VBNC state, 0.25 to 1 ml samples (containing <0.025 or <0.1 CFU, respectively) were removed, placed at 22°C overnight, and monitored for resuscitation by plating onto HI agar. In addition, 0.1 ml samples of the VBNC microcosm were spread plated onto an HI plate to which 200 units of filter-sterilized catalase (C-9322; Sigma, USA) had been previously applied. All plates were incubated at 37°C overnight and monitored for the appearance of colonies.

Results and Discussion

Cells incubated at 5°C in 20% ASW microcosms (190 mOsm) were no longer culturable (<0.1 CFU/ml) after 15 to 28 days, depending on the strain (Fig. 1). On average, *V. parahaemolyticus* cells, regardless of KP type, entered the VBNC state by 20 days in this diluent (Table 2). In contrast, cells in full-strength ASW microcosms (920 mOsm) required a considerably shorter time (average of 7 days) to reach nonculturability (Table 2). Regardless of the diluent or time required to achieve nonculturability, however, approximately 2-4% of the populations were shown to be viable using the substrate responsiveness assay. These data indicate that *V. parahaemolyticus* cells enter into the VBNC state when exposed to a temperature downshift to 5°C, regardless of salinity. As seen in Table 2, *V. parahaemolyticus* strains incubated in 20% ASW (similar to coastal/estuarine salinities) required nearly three times longer to enter the VBNC state as cells incubated in ASW (salinity similar to the open ocean).

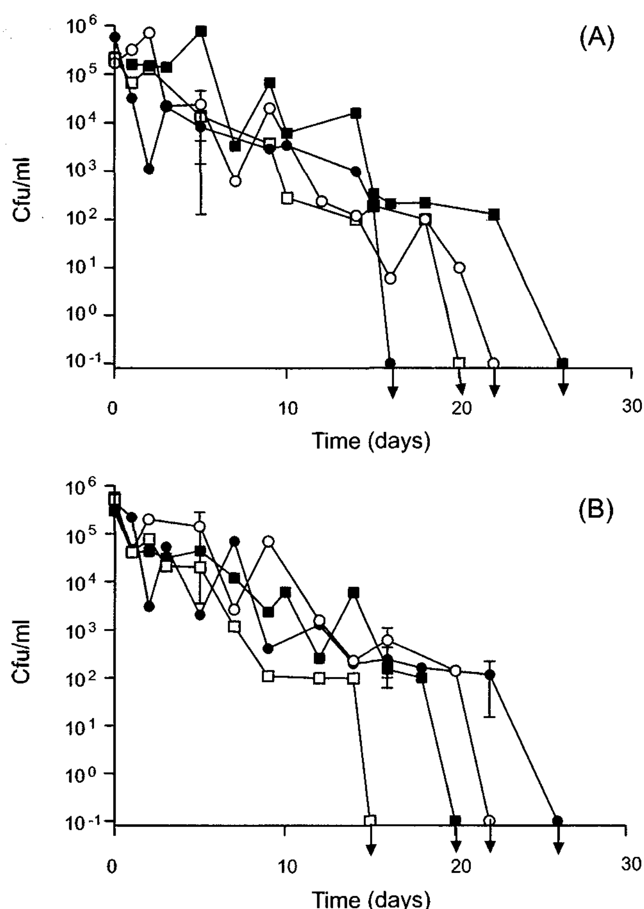

Fig. 1. Results of duplicate studies on the loss of culturability of *V. parahaemolyticus* at 5°C after a nutrient downshift in 20% ASW. Arrows indicate culturability below the limit of detection (10^{-1} CFU/ml). A. KP⁺ strains: ●, SAK11; ○, F11-3A; ■, NY477; □, SPRC851. B. KP⁻ strains: □, AQ4037; ○, VP99; ●, WR1; ■, 1904653.

As shown in Fig. 1, considerable variability was noted in the time that it took *V. parahaemolyticus* strains to enter into the VBNC state. In repeat experiments, times of entrance varied considerably (Table 3). Numerous other studies have also reported such variation in the time that precedes entrance into the VBNC state, and indeed such variation seems to be a hallmark of the VBNC state [Oliver, 2000a, 2000b]. In the case of *V. parahaemolyticus*, Jiang and Chai (1996) examined one KP⁺ and one KP⁻ strain of *V. parahaemolyticus*, and reported times of entrance into the VBNC state to vary from 35 to ca. 80 days. Wong *et al.* (2004) examined 20 clinical and 4 environmental strains of *V. parahaemolyticus*. They observed times to nonculturability of 35-49 days. The times reported in both of these studies were considerably longer than we observed. Whether the increased duration of culturability was due to strain differences, culturing differences, or the method of inducing nonculturability, is not known. Cell preparation was considerably different in our studies, however, with both Jiang and Chai (1996) and Wong *et al.* (2004) employing physiologically older cells which were washed, at a considerably higher initial cell densities (ca. 10^8-10^9 CFU/ml) in the microcosms, and at lower (3.5-4°C) incubation temperatures. The age of cells is known to significantly alter the time required for entry into the VBNC state (Oliver *et al.*, 1991), and we have

Table 2. Average time (range) in days to nonculturability for KP⁺ and KP⁻ strains

Diluent	KP⁺	KP⁻
ASW	6.9 (6-22)	7.2 (4-16)
20%ASW	19.9 (16-28)	20.3 (15-28)

Table 3. Average time (days) to the VBNC state for individual *V. parahaemolyticus* strains in ASW

Strain	Range	Average
NY477	3-9	5.8
SPRC851	7-21	11.9
SAK11	3-8	5.4
F11-3A	4-16	7.5

observed (unpublished data) that washing of *V. vulnificus* cells prior to temperature downshift can also affect VBNC entrance time.

There were no clear differences observed for the time required for KP$^+$ strains versus KP$^-$ strains to enter into the VBNC state (Fig. 1). KP$^+$ cells required, on average, 6.9d to enter the VBNC state when present in ASW, with KP$^-$ cells requiring 7.2d in this diluent (Table 2). In 20% ASW, the same cells required 19.9 and 20.3d, respectively. Indeed, examination of strain AQ3815 and the two co-isogenic strains (Fig. 2) verified that the potential to produce hemolysin does not affect the time it took these strains to enter into the VBNC state. Average times for entry were 15, 14.5, and 16d for the wildtype, *tdh* mutant, and *tdh*-plasmid strain, respectively. It is not known, however, if these organisms were producing hemolysin (s) while in the VBNC microcosms. These results are in contrast to those of Jiang and Chai (1996). They reported that the KP$^-$ strain they examined lost culturability more slowly than the KP$^+$ strain studied. However, as those authors studied only a single strain of each biotype, and given the large differences cells exhibit in time of entry into the VBNC state, such a conclusion seems premature. Indeed, in a study of 24 clinical and environmental *V. parahaemolyticus* strains, Wong *et al.* (2004) concluded that entry into the VBNC state "was unrelated to the source or virulence of these strains". Similarly, our results indicate that differences in retention of culturability can not be ascribed to the presence of the *tdh* gene (s).

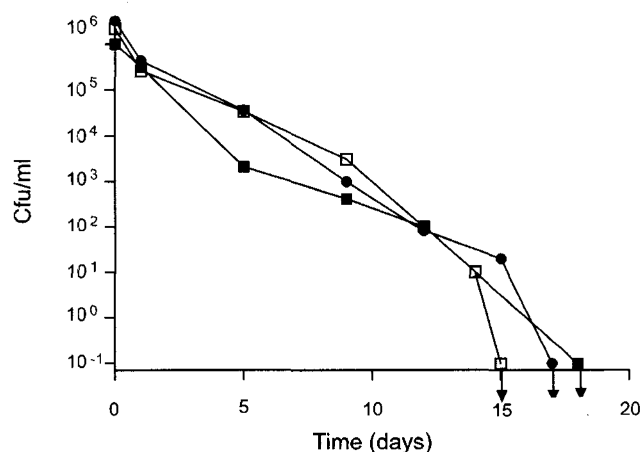

Fig. 2. Loss of culturability of the KP$^+$ parent strain AQ3815 (■), AQ3815Δ*tdh1*Δ*tdh2* (□), and AQ381Δtdh1Δtdh2 pKTN115 (●) following incubation at 5°C in ASW.

Similar to *V. vulnificus* (Whitesides *et al.*, 1997), *V. parahaemolyticus* regained the ability to form colonies following an overnight temperature upshift to 22°C without addition of any exogenous substances. Table 4 shows the results of resuscitation experiments using the 11 strains present in ASW microcosms. Eight of the 11 strains, whether of the KP$^+$ or KP$^-$ biotype, resuscitated to a level within ca. 1 log of the starting cell density following this treatment. Similar results were observed for cells incubated in 20% ASW (data not shown). Our findings are similar to those recently reported by Wong *et al.* (2004). They observed resuscitation when *V. parahaemolyticus* underwent a temperature upshift from 4 to 25°C, but not when shifted to 37°C.

Some researchers have suggested that the apparent resuscitation of VBNC cells is actually due to the regrowth of a few undetected culturable cells remaining in the sample. Indeed, Jiang and Chai (1996) concluded that the cells they recovered after a temperature upshift were likely a result of regrowth of a few surviving cells. We feel that regrowth was very likely in their resuscitation study, in that they employed 5 ml culture volumes which, at 1 CFU/ml, should have contained several culturable cells. To eliminate this possibility, our resuscitation studies were not attempted until filtration indicated <0.1 CFU/ml, with resuscitation volumes of only 0.25 to 1 ml being employed. Using this protocol, our resuscitation studies involved a total

Table 4. Resuscitation of *V. parahaemolyticus* from VBNC (ASW) microcosms

Strain	Kanagawa Phenomenon	Starting Cell Density (CFU/ml)	Cell Density When VBNC (CFU/ml)	Cell Density Following Upshift[a] (CFU/ml)
SAK11	+	2.7×10^5	<0.1	2.6×10^5
F11-3A	+	2.7×10^5	<0.1	1.3×10^3
NY477	+	3.1×10^5	<0.1	1.6×10^5
SPRC851	+	3.3×10^5	<0.1	2.0×10^5
AQ4037	–	8.5×10^5	<0.1	2.3×10^5
VP99	–	6.0×10^5	<0.1	1.7×10^6
WRI	–	3.5×10^5	<0.1	1.6×10^6
1904653	–	3.5×10^5	<0.1	3.4×10^3
AQ3815	+	5.7×10^5	<0.1	3.3×10^3
AQ3815Δ*tdh1*Δ*tdh2*	–	4.2×10^5	<0.1	2.4×10^4
AQ3815Δtdh*1*Δ*tdh2*(pKTN115)	+	3.2×10^5	<0.1	2.0×10^4

[a]Temperature upshift experiments were conducted 24-48 h after the cells were determined to be in the VNBC state.

of <0.025-<0.1 culturable cells, and thus the culturability we observed could not be a result of regrowth of residual culturable cells in these populations, but was due to true resuscitation of nonculturable cells. A similar finding was reported by Wong *et al.* (2004), who conducted their resuscitation studies in a salts mixture (similar to our ASW) which precluded cell proliferation. In our studies, the ability to resuscitate the cells did not persist, with a temperature upshift being no longer effective in restoring culturability of any of the strains after they had persisted in the VBNC state for 1 week. Wong *et al.* (2004) reported that resuscitation of *V. parahaemolyticus* from the VBNC state was strain dependent, but that resuscitation was not observed in 16 of the 20 strains they studied after 7 days of nonculturability.

In our study, when cells in the VBNC state were removed from a microcosm and plated directly onto media supplemented with the ROS scavenger catalase, no growth was observed. The same result was reported by Wong *et al.* (2004), but are in contrast to Mizunoe *et al.* (2000) who reported that media amended with catalase or superoxide dismutase allowed for "resuscitation" of *V. parahaemolyticus* from the VBNC state. Strain diversity or variations in diluents may account for differences observed. Other ROS scavengers (*e.g.* superoxide dismutase) may be appropriate for these strains and should be investigated. However, similar to the findings reported here, Mizunoe *et al.* (2000) found that once cells had been in a nonculturable state for 10 days, no resuscitation was observed on plates amended with ROS scavengers.

Our study failed to provide insight into why differences are observed in the isolation of KP^+ versus KP^- strains from the environment. A study by Pace and Chai (1989) hypothesized that even though fewer KP^+ strains are present in the environment, they are better able to proliferate once they encounter a host. That study demonstrated that the addition of bile to a routine bacterial medium enhanced the growth of KP^+ strains, but not KP^- strains of *V. parahaemolyticus*. They also found that bile supplements increased capsule production, adherence to epithelial cells and Congo red binding. Their observations do not explain, however, why so few KP^+ cells are seen in the natural environment in the first place, and further studies will be required to reveal what accounts for the dramatic difference in the ratio of KP^+ and KP^- cells in the environment.

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