environments. Biofilm formation is linked to development and differentiation in microorganisms at large, possibly also more broadly to multicellular biological differentiation systems (2). Development is not restricted to so-called higher organisms. Developmental processes in bacteria include differentiation of a single cell, such as the swarmer-to-stalk transition by *Caulobacter crescentus* and spore formation by *Bacillus subtilis.* As we learn more about microbial biofilm formation, it is becoming clear that this is yet another example of a bacterial developmental process (35, 36). Like other developmental systems, building a biofilm requires a series of discrete and well-regulated steps. These stages include attachment of cells to a substrate, the growth and aggregation of cells into microcolonies, and the maturation and maintenance of architecture (37, 38). While the stages of biofilm development appear to be conserved among a wide range of microbes, the exact molecular mechanisms may differ from organism to organism. One such different mechanism has been observed in *V. cholerae* that biofilm formation is associated with phase variation of colony morphology (12-14).

Phase variation of *V. cholerae* in response to stress conditions

V. cholerae is the causative agent of cholera, which in its most severe form is characterized by profuse diarrhea, vomiting, and muscle cramps. *V. cholerae* strains have been divided into two groups, O1 and non-O1, based on their ability to cause cholera epidemics. To date, there have been seven recorded pandemics of this severe dehydrating diarrheal disease caused by *V. cholerae* strains of serotype O1, and it was therefore assumed that only this serotype has epidemic potential. The new serogroup, designated O139 synonym Bengal, is the first recorded serogroup other than O1 to cause epidemic cholera. *V. cholerae* O139 closely resembles *V. cholerae* O1 biotype El Tor strains of the seventh pandemic (39-42). The major differences between *V. cholerae* O139 and O1 are the composition and lengths of the O side chains of the cell wall lipopolysaccharide (LPS) and the presence of a capsular polysaccharide (CPS) in O139 strains that is not found in *V. cholerae* O1 strains (43-45). Serological and genetic studies suggested that CPS of O139 *V. cholerae* has the same repeating unit as the O antigen (46, 47).

We have shown that *V. cholerae* O1 and *V. cholerae* O139 underwent phase variation from smooth colony to rugose colony when incubated at low temperature under starvation (Fig. 1). We found that several other stress conditions including high osmorality, high concentration of antibiotics, acidity and freezing can also convert *V. cholerae* to rugose phase variant. The rugose form of *V. cholerae* was first described in 1938 by Bruce White, who recognized that it might serve as a survival form of the organism (48). Rice et al. (49) suggested that the *V. cholerae* rugose phenotype represents a fully virulent survival form of the organism that can persist in the presence of free

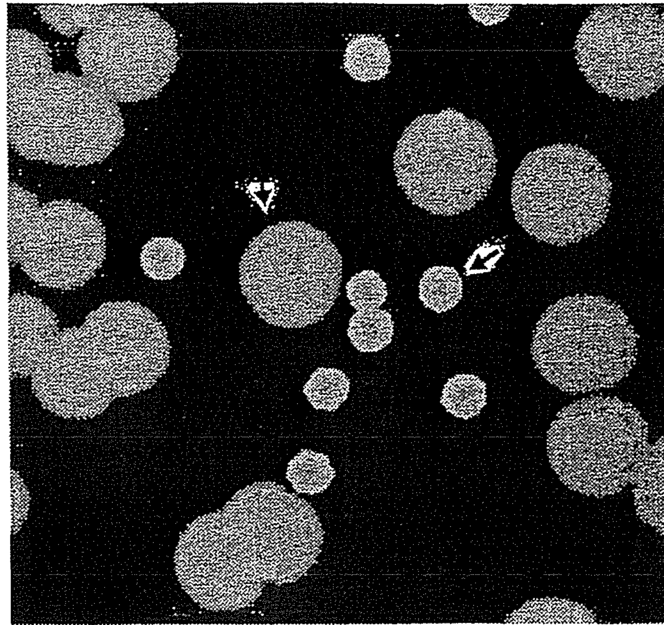

Figure 1. Photomicrograph of *V. cholerae* O139 rugose (arrow) and smooth (arrow head) colonies.

chlorine and that this phenotype may limit the usefulness of chlorination in blocking the endemic and epidemic spread of cholera. Morris et al. (50) subsequently showed that the rugose variant can cause cholera in orally challenged human volunteers and retains its colonial morphology after passage through the human host. More recent studies have shown that epidemic strains of *V. cholerae* switch from the smooth to the rugose phase more frequently than clinical isolates (51), and that this phase transition increases the resistance of the organism to osmotic, oxidative and acidic stress (12-14, 52).

EPS production and biofilm formation by rugose variant of *V. cholerae*

Rugose colony-forming *V. cholerae* O1 produces EPS materials recognized as a heavy, fibrous, electron-dense, ferritin-stained layer surrounding the cells (Fig. 2A), but smooth colony-forming *V. cholerae* O1 did not appear to have this EPS layer surrounding it (Fig. 2B). Like *V. cholerae* O1 rugose strain, *V. cholerae* O139 rugose strain was also shown to produce EPS recognized as heavy electron-dense layer surrounding the cell in addition to a thin electron-dense layer of capsule (Fig. 2C and D). It has been reported that exoploysaccharide (EPS) synthesis is involved in the formation of three-dimensional biofilm architecture in several bacterial species; EPS colanic acid in *Escherichia coli* (53) and EPS alginate in *Pseudomonas aeruginosa* (54, 55).

To determine whether EPS production of *V. cholerae* is related to biofilm formation, *V. cholerae* O1 and O139 rugose variants were cultured overnight

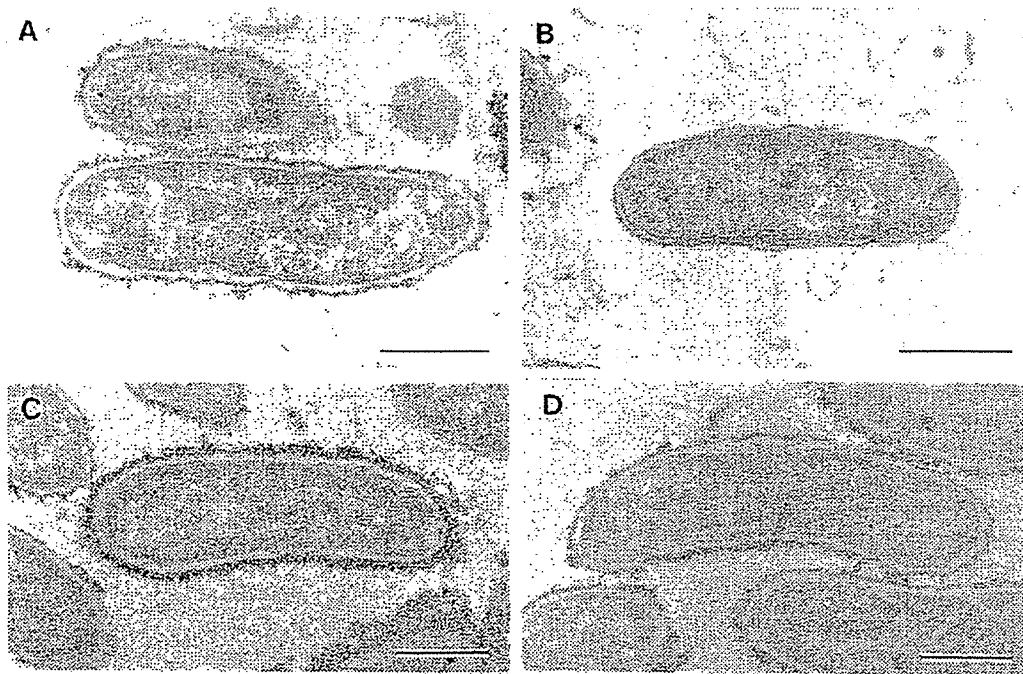

Figure 2. Thin section of *V. cholerae* O1 and O139 stained with polycationic ferritin. O1 rugose strain was surrounded by a thick, electron-dense slime layer (A) and the absence of this layer on O1 smooth strain (B). A thick, electron-dense slime layer in addition to a thin electron-dense layer of capsule on O139 rugose cells (C) and no slime layer on O139 smooth strain (D). Bars, 0.5 μm.

in liquid medium at static condition. We found that rugose variants produced a continuous biofilm on the colonized surface and culture tube walls. The biofilm of rugose variants of *V. cholerae* was clearly visible on the surface of medium and culture tube wall after static incubation, whereas smooth variants did not have the biofilm-forming property and produced a homogenous suspension of bacteria (Fig. 3). Scanning electron microscopic examination revealed that the surface of the biofilm was completely covered with a layer of rod cells embedded within a polymeric matrix (Fig. 4A). Throughout the biofilm, cells were interconnected by a finger-like glycocalyx matrix that extended from the substratum to the outer boundaries of the biofilm. Interestingly, some of the surface of the biofilm was covered by a twisting long filamentous growth of bacteria (Fig. 4B). To investigate the ultrastructural changes of bacteria in biofilm and planktonic counterparts, we sampled biofilm and culture media containing planktonic cells from the same tube after 2 days incubation. Thin-sectioning electron micrographs of *V.cholerae* cells in biofilm revealed the typical rod-shaped cells with a uniform distribution of electron-dense ribosomal and nucleic acid material through out the cytoplasm, seeming to be normal (Fig. 5A). On the other hand, thin-section micrograph of planktonic *V. cholerae* cells from culture media showed that most of cells were

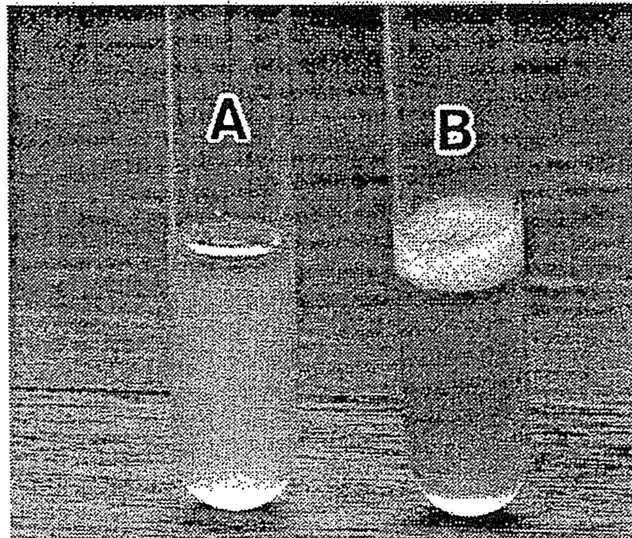

Figure 3. Homogenous bacterial suspension of *V. cholerae* O139 smooth strain in a static culture (A) and biofilm formation of rugose O139 under the same culture conditions (B).

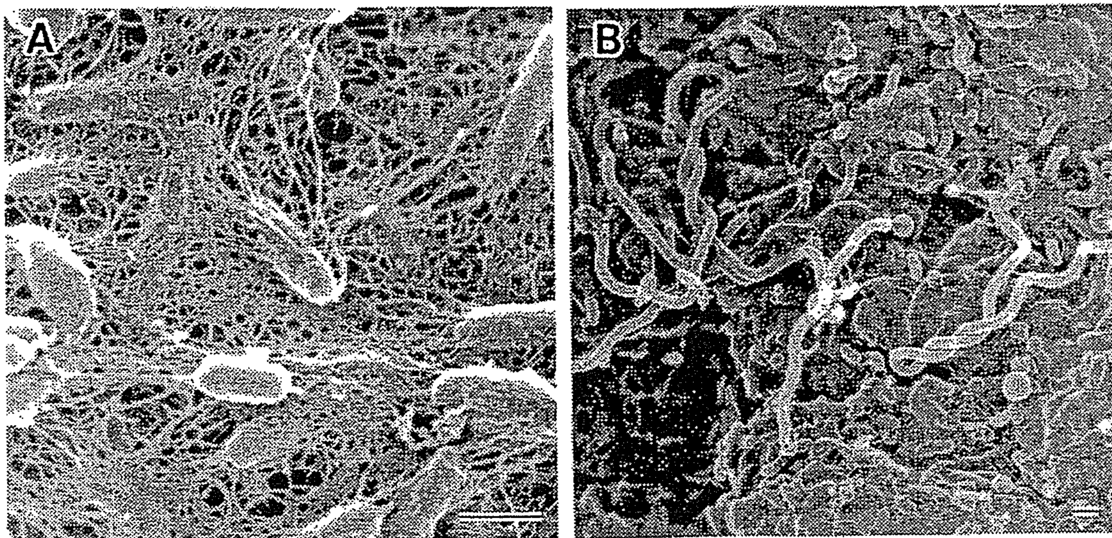

Figure 4. Scanning electron micrographs of biofilm formed by *V. cholerae* rugose strain. (A) Most of the surface has been colonized by rod cells, and finger-like projections of extracellular polymeric material are present. (B) The presence of twisting filamentous or rounded cells on biofillm. Bars, 1 μm.

rounded and contained less internally staining material and probably lost integrity. These cells were probably dead after only 2 days incubation (Fig. 5B). Bacteria in biofilm maintained the normal ultrastructure after 12 days incubation without addition of new nutrient (Fig. 5C). In a murine infection model, it was shown that uropathogenic *E. coli* (UPEC) is able to replicate intracellularly, forming highly organized biofilm-like community of coccoid

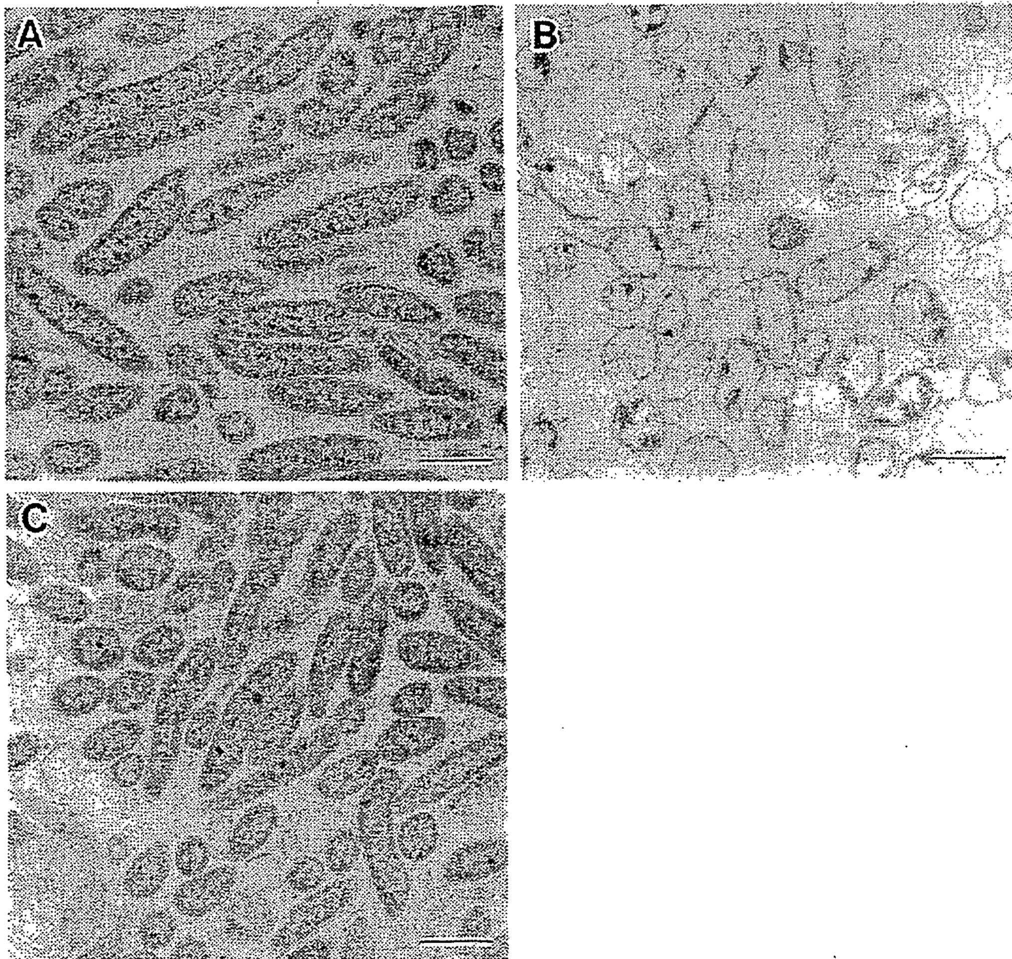

Figure 5. Thin-sectioning electron micrographs of *V. cholerae* cells. Cells in 2 days old biofilm, rod-shaped, with a uniform distribution of electron-dense ribosomal and nucleic acid materials throughout the cytoplasm (A). Two days old planktonic cells, mostly rounded, containing less internally staining materials (B). Cells in 12 days old biofilm showing the normal ultrastructure (C). Bars, 1 μm.

bacteria that ultimately filled most of the cytoplasm, creating a bulge on the bladder surface giving the appearance of a pod (24, 56). Bacteria within the superficial umbrella cells can escape into the bladder lumen in a process termed fluxing. Often, these fluxing bacteria are filamentous (56). Recruited polymorphonuclear leukocytes (PMNs) fail to gain access to the intracellular bacteria and to pagocytose the filamentous bacteria. Intracellular growth and filamentation provided an advantage to the bacteria in evading infiltrating PMNs (26). Biofilm formation and biofilm related morphological change such as filamentation appear to confer the advantage to survive the stress conditions in both host and external environments to organisms.

Molecular genetic analysis of *V. cholerae* biofilm formation demonstrated that EPS production requires a chromosomal locus, *vps* (*Vibrio* polysaccharide synthesis), that contains sequences homologous to carbohydrate biosynthesis

genes of other bacterial species (14). Mutations within this locus yield chlorine-sensitive, smooth colony variants that can not form biofilm. *vps* genes are clustered in two regions in the *V. cholerae* chromosome (14). One cluster harbors genes *vpsA* through *vpsK*, and the other one harbors genes *vpsL* through *vpsQ*. Rugose variants lacking *vpsA* or *vpsL* do not produce EPS and exhibit a smooth colonial morphology (14). Several invesigators have shown the existence of two positive regulators of *vps* genes, VpsR (57) and VpsT (58), and two negative regulators, HapR (60, 61) and CytR (62). Yildiz et al. (63) identified 124 differentially regulated genes by microarray expression profiling studies of the rugose and smooth variants of the same strain. Bioinformatics analysis of these expression data shows that 'rugosity' and 'smoothness' are determined by a complex hierarchy of positive and negative regulators, which also affect the biofilm, surface hydrophobicity and motility phenotypes of *V. cholerae* (63).

An immunoelectron microscopic examination demonstrated that there is an epitope common to the exopolysaccharide antigen of *V. cholerae* O1 and that of O139 (13). The antiserum against *V. cholerae* O1 EPS (12) was reactive only with *V. cholerae* O139 rugose variant and not with smooth variant (Fig. 6). The gold particles were specifically bound to the slime layer surrounding

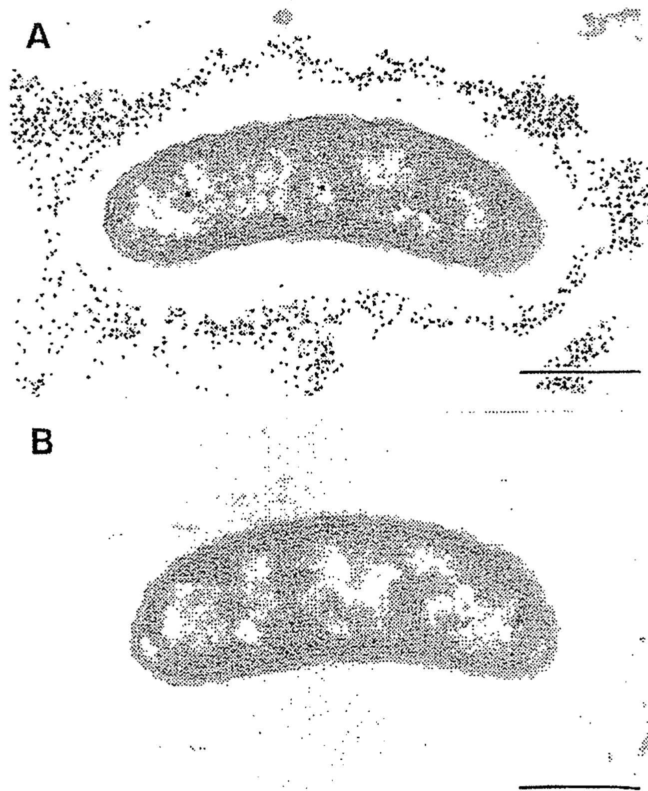

Figure 6. Immunoelectron micrographs of the surface labeling of *V. cholerae* O139 rugose (A) and smooth (B) strains with antiserum against EPS of rugose *V. cholerae* O1. Bars, 0.5 μm.

rugose cells and at the intercellular spaces (Fig. 6A). *V. cholerae* O139 is replacing O1 strains in some areas, and it has been suggested that the O139 strain may cause the eighth cholera pandemic (64, 65). *V. cholerae* O139 Bengal is the second most common etiologic agent of cholera, and the disease caused by this organism has now become endemic in the Indian subcontinent and neighboring countries (66). Prior infection with *V. cholerae* O1, the traditional causative agent of cholera, does not cross-protect against infection with *V. cholerae* O139 (67, 68), since the LPS antigens of the two vibrios are different (44). Vaccines against O1 strains have been developed and are being tested in field trials (69, 70), and they do not cross-protect against *V. cholerae* O139 infection. In our study, interestingly, antiserum against the EPS of *V. cholerae* O1 showed a cross-reaction with EPS materials on the surface of rugose *V. cholerae* O139. We suggest that the study of the genes encoding the EPS (slime) in *V. cholerae* O1 and O139 may facilitate the development of vaccines effective against both *V. cholerae* O1 and O139.

Viable but nonculturable (VBNC) state

A bacterium in the VBNC state is defined as a cell which can be demonstrated to be metabolically active, while beeing incapable of undergoing the sustained cellular division requied for growth in or on a medium normally supporting growth of the cell (28). A variety of environmetal parameters have been reported to induce the entry of various bacteria into the VBNC state (71). Bacteria in VBNC state have been a major concern in public health risk assessment since many pathogenic gram-negative bacteria, such as *V. cholerae, Vibrio vulnificus, V. parahaemolyticus*, and *E. coli* has been shown to enter a VBNC state from which they escape detection and are able to resuscitate to the infectious state following, for example temperature upshift or animal passage (72-74). However, the concept of the VBNC state as a programmed and adaptive response to nutrient starvation has been controversial (29), and another model suggests that cells become nonculturable due to cellular deterioration and, consequently, are moribund (75-77).

The frequency of isolation of *V. cholerae, V. vulnificus,* and *V. parahaemolyticus* is much lower during the winter than the summer months (78). Colwell et al. (79), however, showed that the organism was present and viable in several aquatic environments from which it could not be cultured. This decrease in culturability is thought to arise from the entrance of these bacteria into a VBNC state (28, 80), as has now been shown for at least 30 bacterial species (80). It is thought that the VBNC state may represent a survival response by non-sporulation bacteria exposed to potentially injurious environmental conditions (28). The stresses of low nutrient and low temperature have been shown to be the main causes of the induction of VBNC stages in some pathogenic bacteria (28, 81). In *V. parahaemolyticus, V.*

cholerae and *V. vulnificus*, the VBNC state is induced by a temperature downshift and resuscitation from this state has been reported after the removal of the temperature stress (80-83). Whether true resuscitation of such cells occurs, however, has long been debated. While Whitesides and Oliver (83) have concluded that VBNC cells do indeed leave this dormant state on removal/reversal of the inducing factor, others have suggested that the culturable population which appears is a result of regrowth of one or more culturable cells in the population which had not been detected when the population was assayed (80-82). Resuscitation procedures performed in liquid media, thus, always evoke much controversy. We have tried to resuscitate several VBNC pathogenic bacteria using different methods other than temperature upshift, and will discuss the physiology of VBNC bacteria.

V. cholerae

V. cholerae is a pathogen causing severe diarrheal disease and its infection sources are surface water of natural environments such as rivers, ponds and wells. In epidemic areas, however, detection of *V. cholerae* from natural environment is not always successful (84, 85). Colwell and co-workers have suggested that *V. cholerae* can enter VBNC state (1).

V. cholerae was introduced into VBNC state after 25 days incubation in low nutrient medium at 15°C. 0.1 ml of the VBNC bacterial suspension was heated at 45°C for 1 min and subsequently plated onto a nutrient agar plate. More than 1,000 colonies were recovered after heat-shock treatment (Table 1). After very short period of heat shock treatment, true resuscitation of VBNC cells was observed but not merely regrowth of a few culturable cells remaining in the starting culture (86). Kell et al. (29) reviewed 31 reports about resuscitaion and considered in only three cases, there is sufficient evidence for existence of a reversible state of nonculturablility in nonsporulating bacteria: resuscitation of *Micrococcus luteus* in the presence of a factor produced by viable bacteria and measured using the MPN assay (87), the conversion of

Table 1. Resuscitation of *V. cholerae* from VBNC state by heat shock

Days	Plate count	After heat shock
25	0	5.0×10^3
30	0	2.3×10^3
35	0	0.15×10^3
86	0	6.0×10^3

0.1 ml of the VBNC bacterial culture, with or without heat shock treatment, was plated on L-agar plate and incubated at 37℃ overnight.

nonculturable *V. cholerae* to platable cells via a short heat shock (86), and *Campylobacter jejuni* resuscitated under MPN conditions (88). The mechanism of heat shock on resuscitation remains to be clarified. When bacteria are exposed to high temperatures, a set of proteins is induced to transiently respond to the environmental changes (89). Some of those proteins may participate in the new growth of *V. cholerae*.

V. parahaemolyticus

Since it was first isolated in 1950 during a food-poisoning outbreak in Osaka, Japan, *V. parahaemolyticus* has been shown to be widely distributed in natural aquatic environments around the world (90) and is a well-known food-borne pathogen causing gastrointestinal disease (91).

Starved *V. parahaemolyticus* cells at 4°C reached the nonculturable stage in about 12 days. The true resuscitation of nonculturable cells of *V. parahaemolyticus* occurred after spreading them onto an agar medium supplemented with H_2O_2-degrading compounds such as catalase or sodium pyruvate (92). The response of the *V. parahaemolyticus* following its incubation in starvation medium at 4°C is shown in Fig. 7. Total cell counts remained constant throughout the 16-day period, while colony counts declined

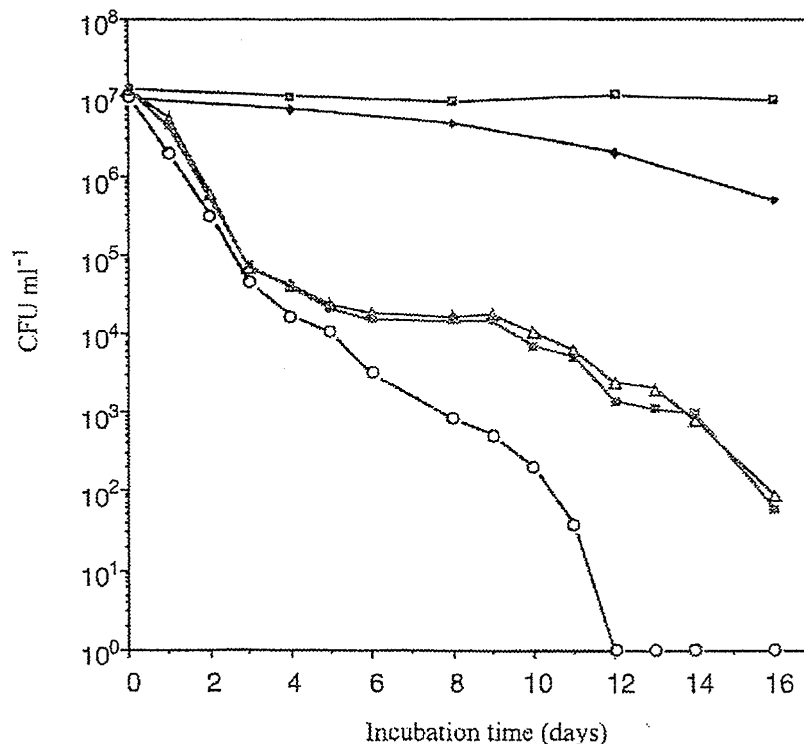

Figure 7. Culturability of *V. parahaemolyticus* incubated at 4°C in the starvation medium (○) and recovery of microcosm samples after spreading on the plates supplemented with catalase (Δ) or pyruvate (■). (□), Total cell counts determined by AODC; ♦, viable counts determined by the Live/Dead staining.

to less than 1 CFU ml^{-1} within 12 days. Despite this decline in colony counts, a significant population of viable cells determined by the Live/Dead staining method remained. After development of the nonculturable state, 0.1 ml specimens of the starvation microcosms were spread onto LB agar-3% NaCl plates supplemented with catalase or sodium pyruvate. After incubation at 37°C for 24 h, nonculturable *V. parahaemolyticus* cells produced a number of colonies with 2.3 x 10^3 CFU ml^{-1} on the plates supplemented with catalase, while no colony was observed on the nonamended plates (Fig. 8, at day 12). The number of colony counts of *V. parahaemolyticus* cells on catalase- or pyruvate-amended plates were always higher than those of nonamended plates. Scanning electron microscopy showed that late exponential phase cells of *V. parahaemolyticus* were normally rod shaped with relatively smooth surface (Fig. 9A). In the VBNC state, starved cells formed blebs on the surface of the rod shaped cells (Fig. 9B). Thin sectioning electron micrographs of late exponential phase *V. parahaemolyticus* cells revealed the typical rod shaped cells with a uniform distribution of electron-dense ribosomal and nucleic acid material through out the cytoplasm (Fig. 9C). Thin section micrograph of nonculturable *V. parahaemolyticus* cells (Fig. 9D) showed that a gap was formed between the outer- and the inner-membrane in some parts. The membrane blebs were observed on some part of the outer membrane. Some cells contained less internally staining material and probably lost integrity. These cells were probably dead (Fig. 9D). Starved cells of *V. haemolyticus* showed the formation of blebs on the surface of the cells. Similar membrane vesicles were observed in other marine organisms, such as *Vibrio* sp. strain Ant-300 and *V. cholerae* during the early stage of starvation (93-95). In the study of Baker *et al.* (96), those cells with a detached cell membrane were viable and responded to nutrient addition as quickly as young healthy cells. The blebs could be formed by pieces of cell envelope. During starvation,

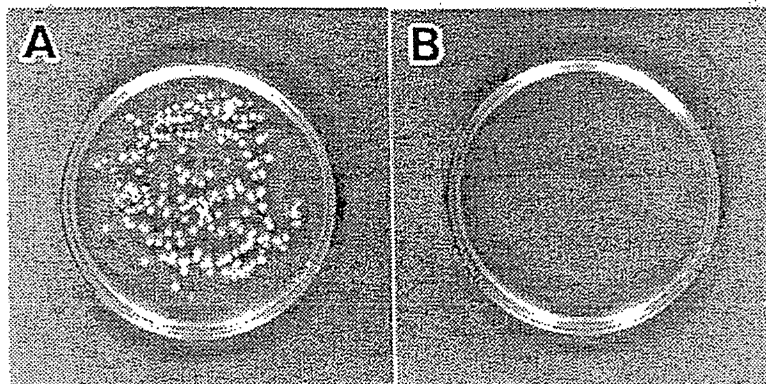

Figure 8. Colonies appeared after resuscitation of nonculturable cells of *V. parahaemolyticus*. Samples (0.1ml) from nonculturable microcosm on day 12 were inoculated on agar amended with catalse (A) or nonamended control (B).

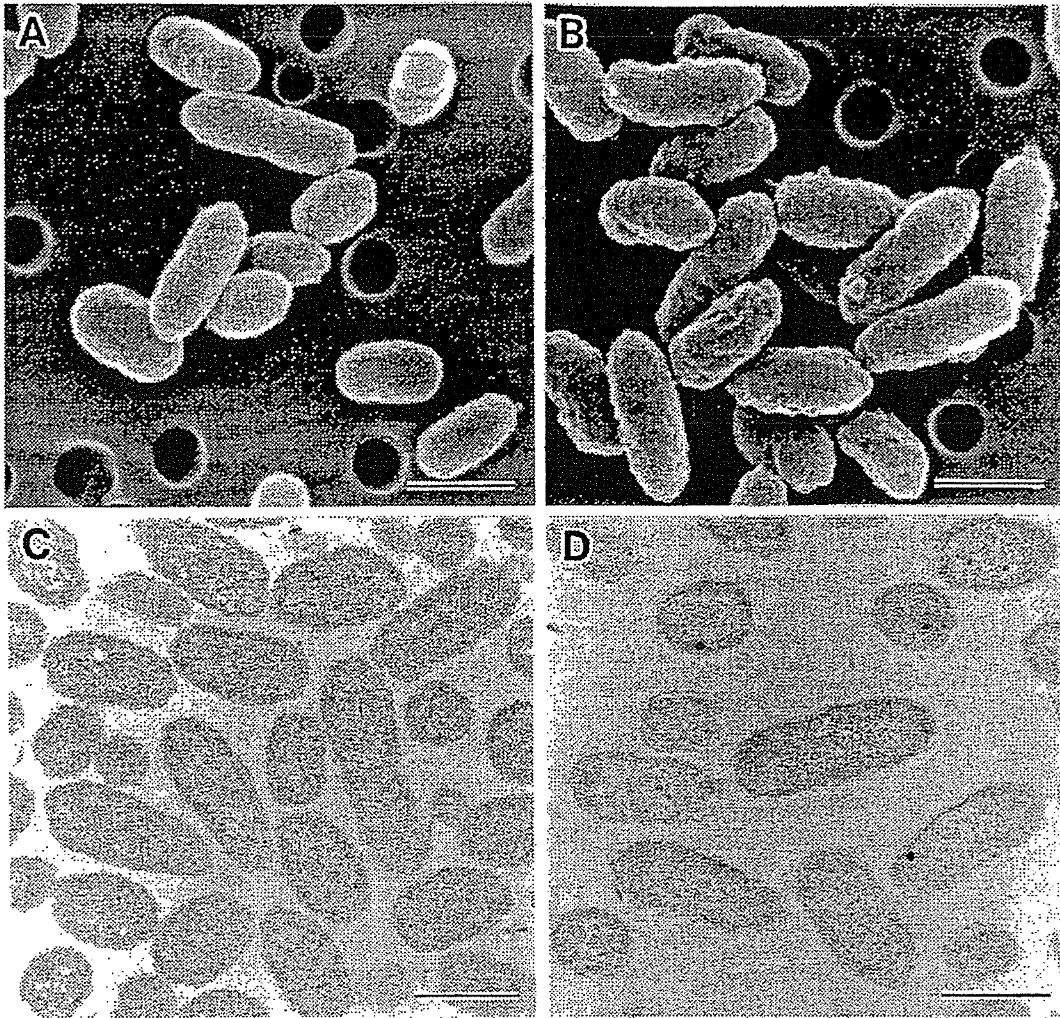

Figure 9. Electron micrographs of *V. parahaemolyticus*. Scanning electron micrograph of late exponential phase cells (A) and cells starved for 12 days at 4°C (B). Thin section electron micrograph of late exponential phase cells (C) and cells starved for 12 days (D). Bars, 1 μm.

excess pieces of cell envelope material may be formed and, in fact, may allow cell volume adjustment via bleb formation. In previous studies, it has been shown that starved cells tend to be small (97, 98). It seems that cell size reduction and bleb formation during starvation is a survival strategy for minimizing cell maintenance requirements and enhancing substrate uptake due to a high surface-to-volume ratio.

Several investigators reported that the viable but nonculturable *Vibrio* species were rounded or spheroid (81, 82, 99). The electronmicrograph of the rounded nonculturable *V. vulnificus* cells (99) showed that the cells maintained a normal cytoplasmic membrane, but possessed a significantly reduced density of ribosomal and nucleic acid material. We suppose that these rounded cells were probably dead. Some studies have argued that resumption of culturability

following a temperature upshift can be accounted for by the regrowth of a few residual culturable cells remaining among the nonculturable population (81, 82, 100). Bogosian et al. (101) demonstrated that the reported resuscitation of VBNC cells during incubation at high temperatures was the result of growth of a small fraction of already culturable, but initially hydrogen peroxide-sensitive, cells. Moreover, there is no evidence for the reductive division exhibited by cells entering the starvation state (6, 79, 102). Taken together, the majority of the nonculturable population prepared in these works (81, 82, 99, 100) does not seem to be alive, then there is no evidence that the rounded cells observed in nonculturable preparations are really viable.

It has been reported that *V. parahaemolyticus* induced into the nonculturable state by low temperature and starvation showed an increased resistance to heating, sonication, low salinity, low pH, or storage at −30°C (82, 103). The VBNC state may make pathogenic *Vibrio* species more resistant to conventional food-processing methods. Besides, virulence may not disappear in VBNC bacteria (79). Seafood is often stored under conditions that induce the VBNC state in *Vibrio* (94). Overreliance on conventional agar plate methods for the microbial inspection of seafoods carries the risk of generating false negative VBNC *Vibrio* species.

Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* (EHEC) is increasingly recognized as a common cause of both epidemic and sporadic disease, notably bloody diarrhea and hemolytic-uremic syndrome. Outbreaks of EHEC infection posed serious health threats in 1996 in Japan (104). Common sources of outbreaks have been traced to the consumption of contaminated hamburger, other foods, and drinking water (105-107). One outbreak-associated isolate of *E. coli* O157:H7 remained viable in tap water for more than a month (108). Furthermore, the unusually prolonged outbreak suggests the survival of these organisms in lake water or repeated contamination but it was not possible to recover the pathogen from the water samples (108). However, this failure to recover the pathogen may result not because of the absence of the organism, but because the cells entered into VBNC state.

Late-exponential-phase cells of *E. coli* O157 became nonculturable in sterilized distilled water microcosms at 4°C. Plate counts declined from 3 x 10^6 to less than 1 CFU ml^{-1} in about 21 days. However, when samples of microcosms at 21 days were inoculated onto an agar medium amended with catalase or nonenzyme peroxide-degrading compounds such as sodium pyruvate or α-ketoglutaric acid, plate counts increased to 10^4-10^5 CFU ml^{-1} (Fig. 10) (Table 2). Like *V. parahaemolyticus*, morphological changes occurred after the cells entered into nonculturable state. Before starvation, late exponential-phase cells of *E. coli* O157 were normally rod shaped with

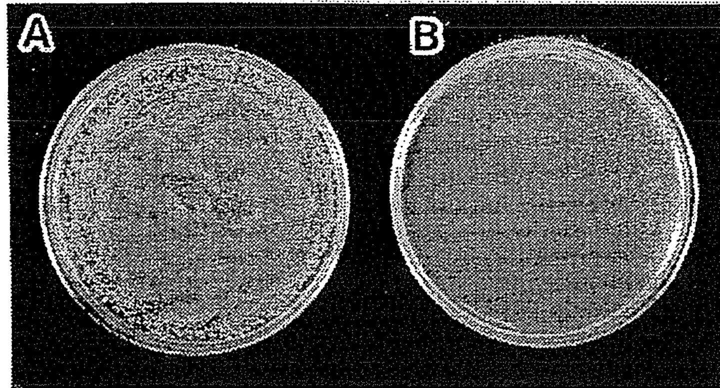

Figure 10. Colonies appeared after resuscitation of nonculturable *E. coli* O157. Sample (0.1ml) from nonculturable microcosm were inoculated on agar amended with catalase (A) or nonamended control (B).

Table 2. Effect of supplements in medium on resuscitation of nonculturable *E. coli* O157 cells. Before resuscitation, plate counts of all samples yielded 0 CFU ml^{-1}.

Supplements for resuscitation	Plate counts (CFU ml^{-1}) After resuscitation
Catalase (2,000U/plate)	1.1×10^5
Sodium pyruvate (0.1%)	2.1×10^4
α-ketoglutalic acid (0.1%)	1.8×10^4
3, 3'-Thiodipropionic acid (0.1%)	4.8×10^4
Heat-denatured catalase*	0

* Originally corresponding to 2000 U/plate of catalase.

relatively smooth surface (Fig. 11A). In nonculturable state, rod-shaped cells appeared to be shorter than that of late exponential-phase cells. The formation of blebs was also observed on the surface of the nonculturable cells (Fig. 11B). Thin-section micrographs of *E. coli* O157 cells (Fig. 11C and D) showed that some parts of the outer membrane of nonculturable cells were separated and a gap was formed between it and the inner membrane.

The infectious dose of *E. coli* O157 is believed to be very low (109). Most of the bacterial populations are probably in the nonculturable state and it seems likely that this state hampers the recovery of the pathogen from the contaminated food and water. This probably explains why the infectious dose of *E. coli* O157 infection is very low. Monitoring disease patterns using laboratory reports is a useful way of looking for major trends, but might be prone to major biases due to under-detection of nonculturable enteric pathogens by routine laboratory methods.

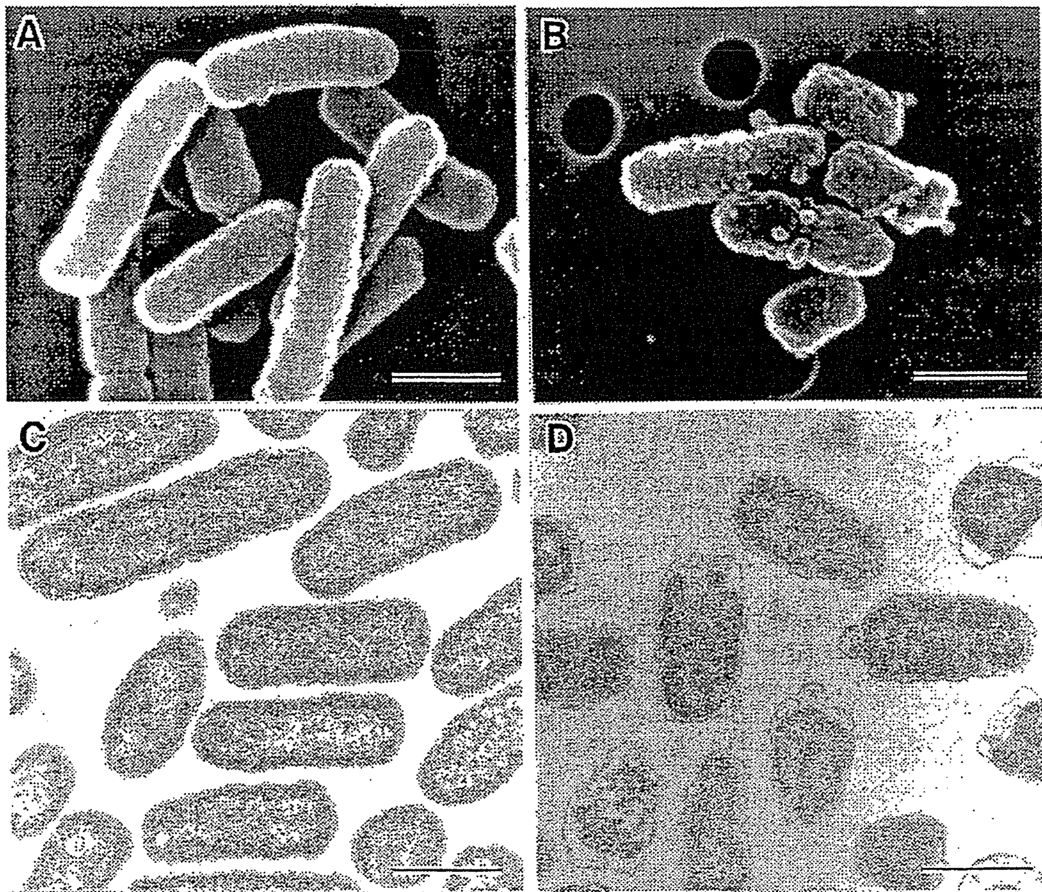

Figure 11. Electron micrographs of *E. coli* O157. Scanning electron micrograph of late exponential phase cells (A) and cells starved for 3 weeks (B). Thin sectioning electron micrograph of late exponential phase cells (C) and cells starved for 3 weeks (D). Bars, 1 μM.

We have demonstrated the resuscitation of VBNC cells of several pathogens including *V. parahaemolyticus* (92), EHEC (110) and *Aeromonas hydrophila* (111) by inoculating onto an agar medium amended with catalase or nonenzyme peroxide-degrading compounds such as sodium pyruvate or α-ketoglutaric acid. We eliminated the incubation process after a temperature upshift of VBNC cell suspension in which regrowth might have occurred, so that the colonies emerging on the supplemented plates are regarded as a consequence of true resuscitation. It is proposed that a sudden transfer of injured or starved cells to nutrient-rich agar at temperatures optimal for enzyme activities initiates an imbalance in metabolism, inducing a near instantaneous production of superoxide and free radicals. In the absence of phenotypic adaptation, the cells are not equipped to detoxify reactive oxygen intermediates and, as a result, a proportion or all of these cells die (112). Our results indicate that an efficient protection against oxidative stress during bacterial inoculation is of fundamental importance in improved recovery of nonculturable cells.

Conclusion

The adaptations that bacteria undergo under stressful conditions are not limited to a few aspects of the cell but rather involve global changes in cell physiology. It is evident that biofilm formation is a key factor for survival in diverse environments. We have shown that *V. cholerae* undergo phase variation to a rugose colony morphology associated with the expression of an amorphous exopolysaccharide that promotes biofilm formation, and we also indicated that rugose strains displayed resistance to osmotic and oxidative stress. Recent advances have led to our current definition of a bacterial biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface. Biofilms constitute a protected mode of growth that allows cells to survive in hostile environments and also disperse to colonize new niches (113).

VBNC state has also been proposed to represent a survival strategy in response to adverse environmental conditions (113). However, it has long been debated whether pathogens exist in VBNC state. The VBNC hypothesis argues for nonculturable cells beeing genetically programmed survival forms awaiting appropriate conditions for re-growth. If VBNC formation is a physiological adaptation similar to spore formation, the mutants that fail to enter VBNC state could be selected (114). No such mutants have been reported. Moreover, almost all published studies fail to discriminate adequately between resuscitation and regrowth of any culturable cells initially present in the population. To eliminate the possibility of such regrowth during resuscitation, we have tried to find new resuscitation methods other than temperature upshift or incubation in some liquid media. In our studies (92, 110, 111), a direct inoculation on the plates amended with hydrogen peroxide-degrading compounds promoted the recovery of several nonculturable pathogens. Our finding was followed by the report of nonculturable *V. vulunificus* (101). We assume that starved nonculturable cells can not be recovered when suddenly transferred to rich medium because of their susceptibility to oxidative stress. We consider such hydrogen peroxide-sensitive condition of cells to be a part of the physiological states of VBNC cells.

Further analysis of molecular mechanisms related to bacterial life cycle including biofilm formation and VBNC state is still of the utmost importance to public health.

Acknowledgements

We thank A. Takade for assistance with the electron microscopic studies.

This work was supported by Grant-in-Aid for Scientific Research (B) (1) 124900009 and (C) (2) 15590391 from the Ministry of Education, Science,

Culture, and Sports of Japan, and grants from Kurozumi Medical Foundation, Ohyama Health Foundation, and Mishima Kaiun Memorial Foundation. Work in SNWs laboratory was supported by grants from the Swedish Research Council, the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), and the Faculty of Medicine, Umeå University.

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