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FEMS Microbiology Letters 180 (1999) 123–131

FEMS MICROBIOLOGY LETTERS

MiniReview

How *Vibrio cholerae* survive during starvation

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Received 20 April 1999; received in revised form 27 August 1999; accepted 30 August 1999

Abstract

Vibrio cholerae, a Gram-negative, motile, aquatic bacterium, is the causal agent of the diarrheal disease cholera. Cholera is a serious epidemic disease that has killed millions of people and continues to be a major health problem world-wide. The hypothesis that *V. cholerae* occupies an ecological niche in the estuarine environment requires that this organism is able to survive the dynamics of physiochemical stresses, including nutrient starvation. As a result of these stresses, bacteria in nature often exist in non-growth or very slow growth states with a low metabolic activity. Because microorganisms have little ability to control their environment, environmental changes have led to changes in cell function and structure. Such cellular responses can originate in one of two ways: by changes in genetic constitution or by phenotypic adaptation. In this review, we will focus on the phenotypic responses of *V. cholerae* of a given genotype to starvation stress.

Keywords: Starvation stress; Rugose colony; Exopolysaccharide; Biofilm; *Vibrio cholerae*

1. Introduction

Environmental stress is a fact of everyday life for microorganisms. Elucidating how microorganisms survive during periods of environmental stress is an exciting area of modern biology. The ability of bacteria to sense and to effectively respond to changes in the environment is crucial for their survival. In general, microorganisms do not respond to nutrient deprivation or starvation by simply arresting all metabolic activity and stopping growth. Instead, they carry out starvation-induced activities that may include production of degradative enzymes, such as proteases and lipases, and substrate-capturing enzymes, such as glutamine synthetase and alkaline phosphatase. In addition, nutrient-deprived bacteria may try to differentiate into a more resistant state to maintain viability during starvation [1–3].

Until the late 1970s and early 1980s, *Vibrio cholerae* was believed to be highly host-adapted and incapable of surviving longer than a few hours or days outside the human intestine. Since then, studies have revealed the existence of *V. cholerae* as free-living bacteria or in association with phytoplankton, zooplankton, crustaceans and mollusks in coastal and estuarine environments [4–7]. These observations, and the capacity of the organism to adaptively re-

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0378-1097/99/$20.00
PII: S0378-1097(99)00454-1

spond to changes in salinity, temperature and the availability of nutrients [5,6], have shown that *V. cholerae* can successfully occupy a variety of aquatic habitats. Although many studies have focused on understanding the starvation response of marine *vibrios*, there is still limited understanding of how *V. cholerae* responds to starvation stress.

2. Gross morphological changes of *V. cholerae* upon nutrient starvation

Non-differentiating bacteria can adapt to nutrient starvation by a variety of genotypic and phenotypic mechanisms [8,9]. It has been proposed that members of Vibrionaceae and Pseudomonadaceae, under conditions of limited exogenous nutrients, can undergo a number of morphological and physiological changes to cope with starvation conditions [10–13]. Starvation-survival has been defined as 'the process of survival in the absence of energy-yielding substrates' [14].

Baker et al. [15] reported that the exposure of *V. cholerae* to nutrient deprivation caused the cells (i) to become coccoid and lose over 90% of their original volume in 30 days, (ii) to increase in cell number, (iii) to lose small granules and inclusion bodies, (iv) to lose the distinct three-layered integrity of the outer membrane, peptidoglycan and the inner membrane to retain only remnants of those structures, (v) to compress the nuclear region into the center of the cell surrounded by a denser cytoplasm and (vi) to form extended or convoluted structures from the cell wall which are pulled away from the cell membrane. It has been suggested that these responses reflect the existence of strategies to enhance survival under condition of exogenous nutrient deprivation [16–18]. For an increase in cell numbers to occur in the initial starvation period, the cells should be able to derive energy from endogenous sources if such cells are nutrient-conditioned before starvation. Additionally, it was noted that nutrient-depleted coccoid-shaped *V. cholerae* cells were restored to normal size and assumed a vibrioid shape within 2 h and began to divide within 5 h after nutrient supplementation. The sustained viability and rapid reversion of coccoid-shaped *V. cholerae* cells to the vibrioid shape and subsequent cell division after nutrient addition may indicate some of the survival mechanisms of *V. cholerae* in the aquatic environment.

Bacteria that have the greatest capacity to survive starvation form small spherical cells develop various stress-resistance functions and have lower metabolic rates than other non-stressed bacteria [19–23]. Dawes and Robbins [24] have compared this characteristic of low metabolic rates with that of spores, which exhibit a capacity for survival by shutting off almost all metabolic activity and yet remain viable. The 'rounding up' phenomenon, with concomitant reduction in cell volume, and other necessary physiological changes are seen for *Vibrio* spp. under low-nutrient conditions [15,25,26].

3. Changes in cellular composition of *V. cholerae* during starvation

Hood et al. [27] reported changes in total cellular carbohydrates and lipids in *V. cholerae* cells in response to starvation. After 7 days of starvation, 88.7% of the carbohydrates and 99.8% of the total lipids had disappeared, followed by smaller additional decreases after 30 days. The rapid disappearance of poly-β-hydroxybutyrate (PHB) was also observed. The rapid disappearance of lipids, carbohydrates and PHB suggests that these compounds are used as energy sources as the cells begin their starvation-survival strategy. Indeed, it would seem a logical strategy for a cell to use the more energy-efficient available compounds (lipids and carbohydrates) as an energy source for dormancy preparation. In their report, RNA, presumably rRNA, levels change very little upon starvation. RNA levels declined only 2% in the first 14 days of starvation and only 20% by 30 days. The DNA concentration and protein levels exhibited a gradual but constant decline, from 30–70% at 7 days to 20–80% at 30 days. It has been suggested that because the protein-synthesizing machinery is so energy expensive, this may be an efficient survival strategy of *V. cholerae*. It might be assumed that the initial reduction in DNA per cell may be related to the increase in cell number during the fragmentation or reduction division stage. However, after the first week of starvation, there was no increase in the number of cells, but there was a continuing decline in the amount of DNA per cell.

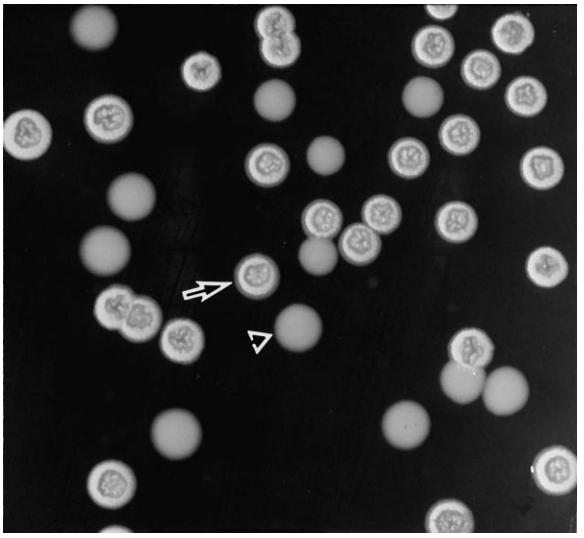

Fig. 1. Photomicrograph of *V. cholerae* O1 rugose TSI-4/R (arrow) and translucent colony TSI-4/T (arrow head).

Whether this puzzling decrease represents a reduction in extra DNA copies, configuration changes in the molecules or some other process is unknown.

Changes in proteins, DNA and RNA in starving *V. cholerae* cells do not duplicate the exact patterns observed in marine *Vibrio* strain ANT-300 [28]. It may be that *V. cholerae* has evolved different cellular strategies that allow for the organism to survive in the estuarine environment with its rapid flux in nutrients rather than the more constant stress of low-nutrients in open ocean waters.

4. Bacterial surface characteristics and the role of an extracellular polysaccharide during starvation

Microorganisms possess a remarkable potential to adjust themselves, both structurally and functionally, to changes in their environment. The alteration in bacterial surface characteristics and adhesion during starvation-survival is of ecological significance. For example, Dawson et al. [29] suggested that such non-growth changes are a tactic in the survival strategy of marine bacteria. They noted the formation of fibrillar structures on the surface of a marine *Vibrio* during the initial phase of starvation. Starvation-induced changes in bacterial surfaces have been reported for several strains of marine bacteria by Kjelleberg and Hermansson [30] and they noted that the increased adhesion and surface hydrophobicity have since been observed as a consequence of the nutritional downshift in marine bacterial isolates.

In our studies [31,32], *V. cholerae* O1 strain TSI-4 and *V. cholerae* O139 strain MO10 could shift to a rugose colony morphology (Fig. 1) associated with the expression of amorphous extracellular polysaccharides (EPSs) in response to nutrient starvation. EPS materials of rugose colony-forming *V. cholerae* strain TSI-4 were recognized as a heavy, fibrous, electron-dense, ferritin-stained layer surrounding the cells (Fig. 2A), but smooth colony-forming *V. cholerae* did not appear to have this EPS layer surrounding it (Fig. 2B). Analogies to rugosity can be found in a number of other species, including the expression of alginate by mucoid strains of *Pseudomonas aeruginosa* and expression of an adhesive EPS by the marine genus *Hyphomonas*. Our reports indicated that cell surface EPS materials confer a rugose colony morphology and resistance to osmotic and oxidative stresses. Owing to the role and properties of EPSs in the adhesion of bacteria to solid surfaces in marine and fresh water environments, studies are often undertaken to identify and characterize EPS-producing marine bacteria [33]. The regulation of EPS synthesis in bacteria is complex and involves multiple systems utilizing both positive and negative regulation [34]. A common feature of all the systems is the participation of two-component regulators consisting of an environmental sensor protein and an effector protein [35–37]. These systems can increase EPS production to meet specific needs of the bacterium during pathogenesis or to enhance its survival in the environment. Global regulatory mechanisms also adjust EPS synthesis according to the general physiological status of the cell, turning it up under nutritional deficiency states or down when carbon and energy must be diverted to growth and repair processes. We have also observed that *V. cholerae* O1 strain TSI-4 and O139 strain MO10 underwent phase variation, converting from translucent to rugose in M9 salts and back again at a frequency of 1.5×10^{-5} in L broth [31,32]. These bi-directional switches between the two colony types would favor outgrowth of the variant best adapted to a particular environment.

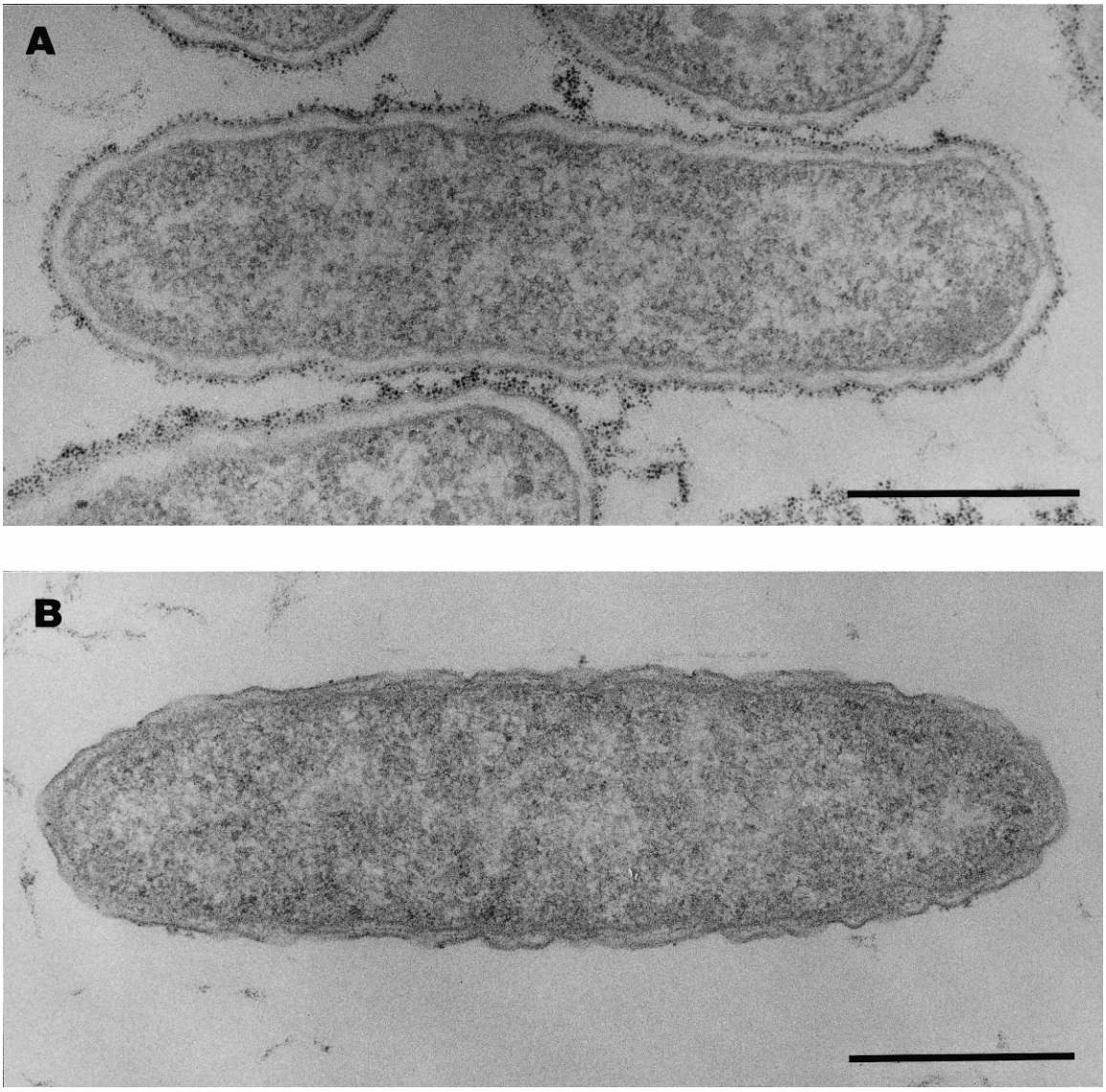

Fig. 2. Thin sections of *V. cholerae* strain TSI-4/R stained with polycationic ferritin showing a thick electron-dense EPS layer completely surrounding the cells (A) and the absence of this layer surrounding the cell of TSI-4/T (B). Bars, 0.5 μm.

5. Rugose survival form of *V. cholerae* during starvation

Rugosity may represent a normal biologic adaptation of a species (*V. cholerae*) that inhabits marine and estuarine environments. The presence of rugose phenotypes of *V. cholerae* has previously been noted but has received little attention since the pioneering studies of White [38]. Rugose strains appear to produce a matrix material, or 'zoogloea', which promotes cell aggregation and which may shield the organism from adverse environmental conditions [39]. The rugose variants were initially reported by Rice et al. [40] to exhibit an increased resistance to chlorination. Also, these strains of *V. cholerae* retained virulence and remained viable at pH 5.0 for 30 min,

suggesting that, under certain conditions, the rugose strain may have a better chance of surviving gastric passage [41,42].

The level of resistance seen with rugose variants (survival in the presence of 2.0 mg l^{-1} free chlorine for up to 30 min) is most unusual for a bacterial pathogen and is more characteristic of the type of resistance seen with extremely resistant forms of microorganisms, such as *Giardia* spp. Chlorination is an effective intervention in controlling cholera [43]. However, if rugose strains are present in a water system, chlorination may simply decrease the number of viable *V. cholerae* but not totally eradicate them. The persistence of viable cholera organisms may then further spread of the infection. The *V. cholerae* rugose phenotype represents a fully virulent survival form of the organism [44] that can persist in the natural environment by using its ability to form biofilms [31,32].

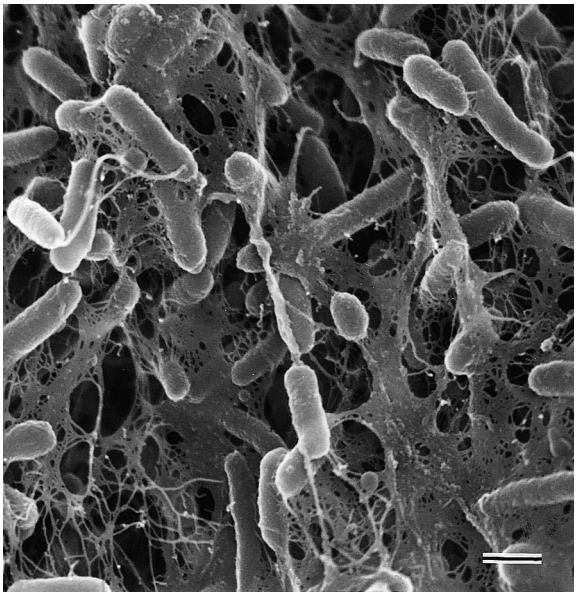

Fig. 4. Scanning electron micrograph of biofilm formation by *V. cholerae* O1 strain TSI-4/R. Most of the surface has been colonized with actively dividing rod cells and finger-like projections of extracellular polymeric material are present. Bar, 1 μm.

6. Biofilm growth of *V. cholerae*

Vibrios are an important component of marine biofilms and it is possible that the EPS produced by *V. cholerae* plays a role in marine biofilm formation. The biofilm in turn may contribute to the attachment of bacteria to marine organisms such as plankton. Within the biofilm, bacteria can access trapped and adsorbed nutrients, engage in favorable metabolic transactions with other members of the biofilm and be protected from predators [45]. It may also provide protection from natural environmental oxidants. The rugose variant is relatively resistant to H_2O_2 [31]. The cells of *V. cholerae* demonstrate a predilection for association with chitinaceous surfaces, the mucilaginous sheath of algae and copepods that can be interpreted as an ecologic advantage [46–49]. In our previous studies [31,32], while examining the morphological characteristics of EPS-producing rugose strains of *V. cholerae*, we found that those strains produced a continuous biofilm on the colonized surface and culture tube walls. The biofilm of rugose *V. cholerae* O1 strain TSI-4 was clearly visible on the surface of L broth and culture tube wall after 5 days of static incubation at 37°C, whereas smooth TSI-4 did not have the biofilm-forming property and produced a homogenous suspension of bacteria (Fig. 3A,B). Under scanning electron microscopy, the surface of the film was completely covered with a layer of contiguous bacterial cells embedded within a polymeric matrix (Fig. 4). Throughout the biofilm, cells were interconnected by a finger-like glycocalyx matrix that extended from

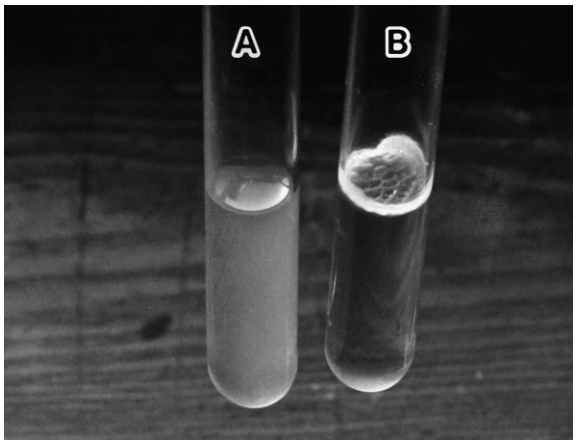

Fig. 3. Smooth bacterial suspension of TSI-4/T in a static culture tube (A) and biofilm formation of TSI-4/R under the same culture conditions (B).

the substratum to the outer boundaries of the biofilm. The glycocalyx appears to bind cells together within the biofilm and ensures the integrity of the biofilm as a whole. Unfortunately, we still know little about the role of *V. cholerae* in forming biofilms, how biofilms may improve survival of this organism in the environment and the epidemiology of the disease in human populations. Furthermore, little is known about the molecular genetic mechanisms affecting biofilm formation by *V. cholerae*. It is important to know whether *V. cholerae* can be identified within biofilms in naturally infected habitats.

7. Starvation and non-culturable state of *V. cholerae*

The 'non-culturable' state of some species of bacteria has been defined as a cell which can be demonstrated to be metabolically active but which is incapable of undergoing the sustained cellular division required to form a colony on media that are regularly used in standard or recommended procedures for bacterial enumeration [50]. Ravel et al. [51], Wai et al. [52] and Colwell [53] suggested that *V. cholerae* O1 possesses the ability to enter a 'viable but non-culturable' (VBNC) state in response to nutrient deprivation, elevated salinity and/or reduced temperature. It has been suggested [54] that the non-recoverability phenomenon can account for the reported sporadic nature of cholera outbreaks because scientists are unable to recover *V. cholerae* from natural waters between epidemics. Clearly, recoverability versus non-recoverability of these bacteria is a phenomenon of critical importance when monitoring the aquatic environment for these potential pathogens.

Aquatic microbial ecologists have long recognized that portions of bacterial populations seem to 'disappear' from natural water bodies at certain times of the year, only to re-appear at other times. There is much to elucidate regarding the VBNC state. The environmental conditions which induce the state probably differ from bacterium to bacterium and it is likely that the physiology involved varies among different cells. *V. cholerae* O1 and *V. cholerae* non-O1 can enter into the VBNC state in response to nutrient deprivation and other environmental conditions [54,55].

Exposure to low-nutrient conditions is an important stimulus to entering the VBNC state [56] and appears to be effective for many different bacterial species. Most studies describing the VBNC state in bacteria have employed laboratory microcosms containing such relatively nutrient-depleted solutions as artificial seawater, river water, M9 salts or tap water. Various Gram-negative bacteria are also known to enter a state of non-culturability, often induced when the bacteria are exposed to adverse environmental conditions. Numerous bacteria other than *V. cholerae*, such as *Escherichia coli*, *Salmonella enteritidis*, *Shigella sonnei*, *S. flexneri, Vibrio vulnificus* and *Legionella pneumophila* [57–60], can enter the VBNC state after exposure to adverse environmental conditions. The existence of VBNC is controversial, since the biochemical parameters which define a cell as viable or dead have not been universally agreed upon [61]. On the other hand, an apparent rapid die-off of *E. coli* has been reported by Bogosian et al. [62]. At the same time, the VBNC phenomenon became puzzling to understand. Barer et al. [63] issued a vigorous call for this frustrating oxymoron to be resolved. Bloomfield et al. [64] have also proposed a plausible model to account, at least partially, for the VBNC paradox. Bogosian et al. [65] developed a mixed culture recovery method to determine whether recovery of culturable bacterial cells from a population of largely non-culturable cells is due to resuscitation of the non-culturable cells from a VBNC state or simply due to growth of residual culturable cells. Their results suggest that the non-culturable cells were dead and that the apparent resuscitation was merely due to the growth of the remaining culturable cells. Kell et al. [66] described some proposals to help to clarify the VBNC paradox. The proposals are of two types: (i) some suggestions concerning operational definitions, together with terms that are best avoided unless strictly defined, and (ii) some suggestions regarding experimental protocols designed to discriminate between some of the major physiological states discussed. We also suggest an alternative terminology that replaces VBNC with expressions that are internally consistent.

8. Conclusions

V. cholerae cells encounter many stresses during

their voyages between natural and host environments. Yet, the organism has proven remarkably versatile in its resilience to these stresses. For its defense, this organism has evolved a complex interconnected series of stress management response systems. In this minireview, we report on the nature of the starvation-survival response of *V. cholerae* with respect to the gross morphological changes, macromolecular synthesis and the development of stress-resistant cells. Nonetheless, relatively little is known concerning the genetic basis and molecular mechanisms of the developmental regulatory process in the starvation-survival response of *V. cholerae*. It is vitally important to know these unknown genetic aspects for the ecology of this organism.

Acknowledgements

We thank Prof. Bernt Eric Uhlin for critical reading of the manuscript. This study was supported by a grant from the Japan Society for the Promotion of Science.

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