The starvation-stress response of *Vibrio (Listonella) anguillarum*

David R. Nelson,¹ Yola Sadlowski,¹ Mitsuru Eguchi²
and Staffan Kjelleberg³

Author for correspondence: David R. Nelson. Tel: +1 401 874 2205. Fax: +1 401 874 7148.
e-mail: dnelson@uriacc.uri.edu

¹ Department of
Biochemistry, Microbiology
and Molecular Genetics,
University of Rhode Island,
Kingston, RI 02881, USA

² Department of Fisheries,
Kinki University,
Nakamachi, Nara 631,
Japan

³ School of Microbiology
and Immunology,
University of New South
Wales, Sydney, NSW 2052,
Australia

The starvation-stress response of *Vibrio (Listonella) anguillarum* was investigated and characterized with regard to changes in cell morphology and the ability of *V. anguillarum* to survive starvation, heat shock, exposure to H₂O₂, and exposure to ethanol. The ability of *V. anguillarum* to survive exposure to the latter three stresses after initiation of starvation was also examined. Results of these experiments indicated that when starved for carbon, nitrogen and phosphorus, the c.f.u. of *V. anguillarum* declined by about one order of magnitude over the first 5–7 d of starvation; starvation for an additional 3–4 weeks resulted in a gradual decline in c.f.u. by another order of magnitude. Examination of starved cells by electron microscopy revealed that while most cells formed spherical ultramicrocells during starvation, some of the cells elongated to form short spirals. While cross-protection against other stresses such as oxidative stress (exposure to H₂O₂) and exposure to ethanol developed, only a small degree of resistance to heat shock developed. Moreover, in all cases these resistances disappeared during prolonged starvation (usually >5 d). Additionally, the rate of protein synthesis per c.f.u., measured by [³⁵S]methionine incorporation, declined during the initial 6 h of starvation and increased to over 70% of the rate measured in exponentially growing cells by 5 d of starvation. It was concluded that the starvation-stress response of *V. anguillarum* differs significantly from those starvation responses reported for other bacteria, including responses displayed by other *Vibrio* species.

Keywords: starvation, *Vibrio (Listonella) anguillarum*, stress response

INTRODUCTION

In natural environments bacteria and other micro-organisms are constantly challenged by a variety of stressful conditions, which include nutrient limitation or starvation, as well as exposure to various forms of physical stress (e.g. temperature variation, osmotic shock, oxidative shock). Some bacteria escape these harsh conditions by differentiating into stress-resistant forms such as endospores or myxospores (Sonenshein, 1989; Zusman, 1984). Recent investigations have demonstrated that many non-differentiating bacteria also have the ability to withstand long periods of nutrient limitation and starvation (Matin *et al.*, 1989; Morita, 1993; Östling *et al.*, 1993; Spector & Foster, 1993). Indeed, it has been suggested that in natural environments most micro-organisms face intermittent or prolonged periods of nutrient-limiting conditions and rarely, if ever, grow at rates approaching maximum

(Moriarty & Bell, 1993; Morita, 1993). Recent investigations indicate that at the onset of starvation many bacteria exhibit the coordinated expression of protective mechanisms that allow the cells to survive exposure to multiple physical stresses (Givskov *et al.*, 1994b; Jenkins *et al.*, 1988; Östling *et al.*, 1993). It is thought that the acquisition of such cross-protection during starvation is the result of the expression of starvation-stress proteins (Givskov *et al.*, 1994a; Hengge-Aronis, 1993). In contrast, Eguchi *et al.* (1996) suggest that marine oligotrophic bacteria grow at relatively constant rates over a very wide range of nutrient concentrations and are resistant to multiple physical stresses during both growth and starvation.

Vibrio species are generally found in the cultivable fraction of marine bacteria (Östling *et al.*, 1993). While members of this genus proliferate in sites of high substrate concentration and reach high cell density, they

are able to persist as free-living organisms in nutrient-limited waters for extended periods of time (Östling *et al.*, 1993). Their coexistence with the dominant pool of oligotrophic bacteria in carbon-limited waters and their appearance in association with higher organisms suggest a successful life cycle of feast and famine. This is perhaps best documented for *Vibrio (Listonella) anguillarum*. This bacterium causes epizootics of vibriosis in a variety of marine fish and shellfish (Austin & Austin, 1993; Bolinches *et al.*, 1986; Bowser *et al.*, 1981), but is able to persist under conditions of carbon starvation for at least four years (Hoff, 1989).

Here we examine the starvation-stress response of *V. anguillarum* with regard to the ability of this bacterium to adapt to and survive starvation and other stresses including heat shock, exposure to ethanol and exposure to hydrogen peroxide. We present evidence indicating that *V. anguillarum* exhibits a response to long-term starvation that is different from those responses described for copiotrophs such as *Vibrio* species strain S14 (Östling *et al.*, 1993), *Escherichia coli* (Matin, 1991) and *Pseudomonas putida* (Givskov *et al.*, 1994b). The latter three species of bacteria exhibit long-term starvation survival strategies that may be characterized as self-contained. That is, cells exposed to starvation respond rapidly and in an ordered, sequential fashion to express genes and proteins that allow starved cells to become relatively less metabolically active and more resistant to a variety of potential environmental insults (Givskov *et al.*, 1994b; Matin, 1991; Östling *et al.*, 1993). In contrast to this response, our data suggest that *V. anguillarum* cells exhibit a somewhat different strategy towards starvation. This alternative strategy does not include a long-term reduction in metabolic activity and long-term stress cross-protection; rather *V. anguillarum* cells first lose and later regain metabolic activity during starvation. Additionally, *V. anguillarum* cells exhibit only a limited set of starvation-acquired cross-protections, which are lost as starved cells regain metabolic activity.

METHODS

Bacterial strains and culture conditions. *V. anguillarum* 2129 was isolated from a diseased fish. Cultures of *V. anguillarum* were routinely grown and maintained in Luria-Bertani broth (Maniatis *et al.*, 1982) plus 2% NaCl (LB20) at 25 °C with shaking or on LB20 agar plates also incubated at 25 °C. Typically for experiments, aliquots from overnight cultures grown in LB20 at 25 °C were transferred to flasks of marine minimal medium (3M) (Neidhardt *et al.*, 1974) and grown to mid-exponential phase to stationary phase (10^7 – 10^9 c.f.u. ml⁻¹). Growth was measured as OD₅₅₀.

Starvation and stress-challenge protocols. *V. anguillarum* cells were starved for carbon (C), nitrogen (N) or phosphorus (P), or all three simultaneously (CNP). Cells to be starved were grown to the desired density (10^7 – 10^9 c.f.u. ml⁻¹), harvested by centrifugation (9000 g, 10 min, 25 °C), and washed and resuspended in the appropriate starvation broth. Starvation for carbon was performed in 3M minus glucose. Starvation for nitrogen was performed in 3M minus NH₄Cl. Starvation for phosphorus was performed in 3M minus potassium phosphate. Starvation for CNP was performed by incubating the

cells in either 3M without added glucose, NH₄Cl and potassium phosphate or in nine salts solution (NSS) (Marden *et al.*, 1985). All starvation experiments were carried out at 25–28 °C. To determine the effects of starvation on the viability of *V. anguillarum* cells, samples were withdrawn at various intervals after the initiation of starvation and the c.f.u. determined by dilution and plating onto LB20 agar or, in some cases, VNSS agar (Nyström & Kjelleberg, 1987). All plate counts were performed in triplicate.

Cells to be heat shocked were harvested by centrifugation, washed twice (in either 3M or NSS), and resuspended in fresh 3M broth or in NSS at 25 °C. Aliquots were withdrawn from the 3M cultures after 5 min of equilibration or from the NSS starvation cultures at the times indicated in the text and transferred to a 42 °C water bath for heat shock. To determine the effects of heat shock on the viability of *V. anguillarum* cells, samples were withdrawn 60 min after transfer to 42 °C and the c.f.u. determined by serial dilution and plating onto LB20 agar. All plating was carried out in triplicate.

Cells to be challenged by exposure to H₂O₂ were harvested by centrifugation, washed and resuspended in 3M broth or NSS as described above. Aliquots were withdrawn and H₂O₂ added to a final concentration of 2 mM. Samples were withdrawn 15 min after the addition of H₂O₂ and the c.f.u. determined by serial dilution and plating onto LB20 agar.

Cells to be challenged by exposure to ethanol were harvested, washed and resuspended in 3M or NSS as described above. Aliquots were withdrawn and ethanol added to a final concentration of 12% (v/v). Samples were withdrawn 15 min after the addition of ethanol and the c.f.u. determined by serial dilution and plating onto LB20 agar.

Electron microscopy. *V. anguillarum* cells were fixed by the addition of 50% glutaraldehyde (EM grade; Electron Microscopy Sciences) to a final concentration of 1% (w/v). For negative stains, one drop of culture was placed onto a 200 mesh C-coated/collodion grid. After 10 min, the drop was removed and the grid was negatively stained with 1% ammonium molybdate. For thin sections, a 5 ml aliquot of each culture was centrifuged at 9000 g for 30 min at 5 °C. The cell pellet was gently disrupted and enrobed in molten (44 °C) 1.5% purified agar in 0.1 M sodium cacodylate buffer (pH 7.2). The excess agar was trimmed away from around the cell pellet fractions and these small agar cubes were placed in 3 ml vials containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4 °C. The samples were washed three times (30 min each wash) with buffer, fixed with 1% osmium tetroxide in buffer for 3 h, and dehydrated in a graded ethanol series to 95% ethanol. All washes and ethanol dehydrations were carried out at 4 °C. The samples were allowed to warm to room temperature and then changed four times with absolute ethanol for 14 min each. After two additional changes with propylene oxide, the samples were embedded in Spurr's epoxy resin and thin-sectioned with a diamond knife on a DuPont/Sorval MT2-B ultramicrotome. The sections were stained with 2% uranyl acetate in 50% methanol followed by lead citrate. The negative stains and thin sections were examined and photographed with a JEOL 1200 EX transmission electron microscope operating at 80 kV.

Radioactive labelling of cells. Concurrent with sampling for viability measurements, 1 ml cell suspension in either 3M (exponentially growing cells) or NSS (starved cells) was labelled with 10 µCi (3.7×10^5 Bq) [³⁵S]methionine (Dupont, NEN Research Products; sp. act. > 600 Ci mmol⁻¹) for 30 min at 25 °C. When the labelling period was complete, the cells were centrifuged (15000 g, 2 min), washed twice in NSS, and

resuspended in 100 μ l 10 mM Tris buffer (pH 7.2), and frozen for storage. Incorporated radioactivity was determined by scintillation counting of TCA-precipitated protein pellets (Nelson & Zusman, 1983).

Gel electrophoresis and autoradiography. One-dimensional SDS-PAGE was performed as described by Laemmli (1970) using 5% stacking gels and 10% separating gels. Autoradiography was performed as previously described (Nelson & Killeen, 1986). Equal volumes of labelled cell suspensions were loaded onto each lane.

RESULTS

Changes in cell viability and morphology during C or CNP starvation

The response of *V. anguillarum* cells to starvation for C was compared to simultaneous starvation for CNP. For periods of starvation up to at least 28 d (672 h), the survival of cells (as measured by c.f.u.) starved for C paralleled that of cells starved for CNP (Fig. 1a). Typically, the drop in c.f.u. over a 4 week starvation was slightly greater than two orders of magnitude. The decline in viable cells was usually biphasic, with the most rapid drop in c.f.u. occurring during the first 72–168 h (3–7 d) of starvation. The decline in c.f.u. slowed after that time. Additionally, over the first 6–24 h of starvation there was often a two- to threefold increase in c.f.u. This general pattern of cell viability was reproducible in starvation experiments with initial cell densities ranging from 10^7 to 10^9 c.f.u. ml^{-1} (data not shown).

The effects of potential oxygen-limiting conditions and the composition of the recovery plating medium on starvation survival were also examined (Fig. 1b). Cells were starved for CNP in either shaking or standing cultures. Samples were withdrawn at intervals and plated onto either a high-concentration nutrient agar (LB20) or a lower-concentration nutrient agar (VNSS). Regardless of the starvation conditions (shaken or still)

and the recovery growth conditions (high or low nutrient), no differences in c.f.u. were detected. Further, an examination of pH during starvation revealed that the pH decreased by only 0.3 unit from pH 7.5 during the 4 week starvation.

The morphology of cells starved for CNP was examined by transmission electron microscopy of both negatively stained cells and thin sections of cells (Fig. 2). During the first 24 h of starvation, some changes from exponential-phase cells to starved cells were observed. The most noticeable differences between exponential-phase cells (Fig. 2a, b; mean diameter $\pm$ SD of 25 cells was 0.54 ± 0.05 μm) and cells starved for 24 h (Fig. 2c, d; mean diameter 0.46 ± 0.06 μm) were a decrease in the observed diameter of about 15% and the appearance of some helical cells. There were few differences between cells starved for 48 h (not shown) and cells starved for 24 h. By 168 h (Fig. 2e, f) of starvation, there were a number of clearly observed changes in the morphology of the cells. Most cells had become rounded, contained less internally staining material and probably lost integrity. These cells were probably dead. From numerous observations by both electron microscopy and phase-contrast microscopy, it appeared that about 10% of the cells appeared to have elongated into curved or helical filaments. Cells that retained an intact cell envelope had a mean diameter about 78% (0.42 ± 0.06 μm) of that observed in exponential-phase cells. Observations by phase-contrast microscopy indicated that most cells lost motility between 6 and 24 h after the initiation of starvation. However, a small number of motile cells could be observed even after prolonged periods of starvation.

Changes in cell viability during starvation for N or P

The effects of starvation for N or P on *V. anguillarum* viability were examined. Typical cell survival curves for *V. anguillarum* starved for N or P (for periods up to

Fig. 1. Survival of *V. anguillarum* under conditions of starvation for C or CNP. (a) Cells starved for C (●) or CNP (■) were compared. Cells were grown to $\sim 10^8$ c.f.u. ml^{-1} in Marine Minimal Medium (3M) at 25 °C, washed, resuspended in the appropriate starvation medium and incubated at 25 °C. Samples were withdrawn at indicated intervals and the c.f.u. determined by serial dilution and plating on LB20. (b) Cells starved for CNP were compared for survival during incubation in shaken cultures (○, △) or stationary cultures (□, ▽). Cultures were prepared as described above and c.f.u. determined by dilution and plating on either LB20 (○, □) or on VNSS (△, ▽).

Fig. 2. Electron micrographs showing the morphology of growing and starved (24 h and 168 h) *V. anguillarum* cells. Cells were grown in 3M (a, b) or allowed to starve for C, N and P for 24 h (c, d) and 168 h (e, f). Panels a, c and e show cells negatively stained with uranyl acetate. Panels b, d, and f show cells thin-sectioned and stained with uranyl acetate and lead citrate. Bars, 1 μ m.

840 h) are shown in Fig. 3. A c.f.u. survival curve for CNP starvation is included for comparison. During the first 24 h of starvation the c.f.u. increased in both N- and P-starved cultures (three- and ninefold, respectively). Subsequently, the c.f.u. for both N- or P-starved cells decreased. The c.f.u. of N-starved cells decreased by 1.1

orders of magnitude (12-fold) between 24 and 120 h. This is about the same decrease as observed in CNP-starved cells between 0 and 120 h of starvation. Between 120 and 840 h (5 and 35 d) of N starvation the c.f.u. declined by an additional 0.6 log. The c.f.u. of P-starved cells decreased by almost three orders of magnitude

Fig. 3. Survival of *V. anguillarum* starved of CNP (●), N (■) and P (▲). Cells were grown to $\sim 10^8$ c.f.u. ml $^{-1}$ in 3M at 25 °C, washed, resuspended in the appropriate starvation medium and incubated at 25 °C. Samples were withdrawn at indicated intervals and the c.f.u. determined by plating on LB20.

between 24 and 120 h. Between 120 and 840 h of P starvation, the c.f.u. appeared to oscillate with a periodicity of 300–500 h between peaks in c.f.u. It is interesting to note that the long-term (> 168 h) c.f.u. survival of *V. anguillarum* was not dependent upon the depleted nutrient.

Starvation-induced cross-protection in *V. anguillarum*

Recently it has been reported that a number of different bacteria develop a multiple stress-resistant state when the organism is starved of nutrients (Givskov *et al.*, 1994b; Jenkins *et al.*, 1988; Matin, 1991; Spector & Foster, 1993). We sought to determine whether this was the case for *V. anguillarum*. Growing cells (mid-exponential phase in 3M) and cells starved in NSS for periods of up to 168 h were challenged with heat shock (42 °C), H₂O₂ (2 mM) or ethanol (12%) (see Fig. 4). The

sensitivity of the cells to each of these treatments was determined by the time-dependent reduction in c.f.u. The data presented illustrate representative experiments from a minimum of three trials for each stress. The general survival patterns described are reproducible. As shown in Fig. 4(a), exponentially growing cells ($t = -1$ h) declined by more than two orders of magnitude when exposed to a 42 °C heat shock for 60 min. Cells starved for up to 120 h were somewhat more resistant than growing cells. However, cells starved for 168 h lost the small amount of resistance gained during shorter periods of starvation and were slightly more sensitive to heat shock than growing cells.

A somewhat different pattern of starvation-induced cross-protection was observed for cells challenged with H₂O₂ (2 mM, 15 min; Fig. 4b). Growing cells ($t = -1$ h) were extremely sensitive to oxidative stress, exhibiting a decline in c.f.u. of almost four orders of magnitude during a 15 min exposure to H₂O₂. Cells washed free of growth medium and resuspended in NSS rapidly gained resistance to H₂O₂. Resistance peaked by 24 h of starvation, with about 50% of the starved cells surviving H₂O₂ exposure. Oxidative-stress resistance exhibited a broad plateau between 24 h and 96 h of starvation. After 96 h, resistance declined and by 168 h of starvation, the cells were almost as sensitive to oxidative stress as growing cells.

A third variation of starvation-cross protection was seen for starvation-acquired resistance to ethanol stress. Growing cells exposed to 12% ethanol for 15 min declined by about two orders of magnitude. However, cells washed free of 3M broth and resuspended in NSS were extremely sensitive to ethanol. Typically, survival was only 10^{-5} of the input cell population. By 24 h of starvation, *V. anguillarum* cells exhibited about the same sensitivity to ethanol as growing cells, with about 1% of the input cells surviving the ethanol shock. Resistance increased about tenfold between 24 h and 72 h of starvation. Ethanol resistance declined after

Fig. 4. Effects of starvation on survival of *V. anguillarum* cells exposed to (a) heat shock at 42 °C; (b) 2 mM H₂O₂; and (c) 12% ethanol. Cells were grown in 3M to $\sim 10^8$ c.f.u. ml $^{-1}$ at 25 °C, washed and resuspended in NSS starvation medium (minus CNP) and incubated at 25 °C. Aliquots were withdrawn periodically to determine starvation survival (○) and to determine stress resistance (■) by challenge with the appropriate stress as described in Methods. C.f.u. were determined by serial dilution and plating on LB20. Each data point is the mean of triplicate samples. Error bars indicate the standard deviation. The data shown are representative experiments from a minimum of three trials for each stress.

Table 1. Relative rates of [35 S]methionine incorporation by *V. anguillarum* starved for CNP

Cells were labelled and radioactivity determined as described in Methods. The relative rates of [35 S]methionine incorporation were determined by dividing the value of the incorporated c.p.m. per 10^8 c.f.u. for any time point by the value determined for exponentially growing cells. The value for [35 S]methionine incorporation in exponentially growing cells was 7.5×10^4 c.p.m. per 10^8 c.f.u.

Duration of starvation (h)	Relative rate of [35 S]methionine incorporation
Exponentially growing cells	1.00
Starved cells	
0	0.58
1	0.47
6	0.17
24	0.28
48	0.31
72	0.46
120	0.70
168	0.73

72 h, and by 168 h of starvation the starved cells were almost as sensitive to ethanol as growing cells.

Protein synthesis by starved *V. anguillarum* cells

The appearance of helical cells and the loss of starvation-induced stress cross-protection during prolonged (> 24 h) starvation suggested that *V. anguillarum* cells were not dormant. To examine this more directly, cells were labelled with [35 S]methionine during exponential growth and at various times during starvation for CNP (in NSS). The relative rates of [35 S]methionine incorporation into TCA-precipitable material were determined and are shown in Table 1. The data reveal that during the first 6 h of starvation the rate of incorporation per c.f.u. fell by over 80% from that of exponential-phase cells. The rate of incorporation per c.f.u. increased after that time, and by 168 h the rate was almost 75% of that observed in exponential-phase cells. Examination of protein expression in growing and starved cells labelled with [35 S]methionine by SDS-PAGE, autoradiography, and densitometry (Fig. 5) revealed a similarity with the data presented in Table 1. Both the number of proteins expressed and the overall level of protein expression fell rapidly from 37 bands detected in growing cells (and $t = 0$ h starved cells) to a minimum of 17 detectable bands with a total density of ~6% that of growing cells in cells starved for 24 h. Both the complexity and the levels of protein expression began to increase by 48 h of starvation. Twenty-three protein bands with a total density of 7% that of growing cells (and $t = 0$ h cells) were detected in cells labelled after 48 h of starvation. By 168 h of starvation, the number of detectable bands had increased to 31 with a total density of about 25% that of growing cells.

Fig. 5. Autoradiogram of [35 S]methionine-labelled proteins from growing and starved *V. anguillarum* cells. Cells were grown in 3M broth and starved in NSS broth as described in Methods. Aliquots were taken and labelled with [35 S]methionine during growth in 3M broth (lane 1) and at the following times during starvation: lane 1, exponentially growing cells; lane 2, 0 h; lane 3, 1 h; lane 4, 6 h; lane 5, 24 h; lane 6, 48 h; lane 7, 72 h; lane 8, 120 h; lane 9, 168 h. Molecular mass standards are indicated in kDa.

DISCUSSION

It is clear that marine and other bacteria have evolved mechanisms to survive the stress of starvation until nutrients again become available to support the growth of cells. The starvation-survival strategies employed by bacteria include at least the adaptive processes of oligotrophy, spore and cyst formation, and copiotrophy (Östling *et al.*, 1993; Poindexter, 1981; Rozak & Colwell, 1987; Schut *et al.*, 1993). Copiotrophic bacteria exhibit relatively rapid growth rates under conditions of abundant nutrients and survive starvation without the development of spores or cysts (Nyström *et al.*, 1990). Such organisms include well-characterized 'non-differentiating' bacteria such as *E. coli*, *P. putida* and *Vibrio* sp. S14. When starved, particularly of carbon or multiple nutrients, these bacteria rapidly form starvation-stress-resistant cells which are generally characterized by their small, coccoid shape (ultramicrocells), their rapid acquisition of cross-protection against a variety of stresses and their instantaneous response to added nutrients (Givskov *et al.*, 1994a,b; Holmquist &

Kjelleberg, 1993; Martin, 1991; Nyström *et al.*, 1990; Östling *et al.*, 1993). In other words, these bacteria survive starvation by expressing genes which enable the cells to enter a starvation-induced adaptation state, which is resistant not only to long-term starvation but also to a variety of physical and chemical stresses, and permits the cells to initiate outgrowth when nutrients are available (Marouga & Kjelleberg, 1996). This response to starvation may be characterized as relatively non-active or self-contained. That is, the cells 'go with the flow (or nutrient flux)'.

In contrast to the strategy employed by copiotrophs such as *E. coli*, *P. putida* and *Vibrio* sp. strain S14, a different response to starvation has recently been documented for the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256 (Eguchi *et al.*, 1996). This marine oligotroph, which displays a remarkably constant maximum specific growth rate ($0.13\text{--}0.16\text{ h}^{-1}$) over a wide range of organic carbon concentrations ($0.8\text{--}800\text{ mg C l}^{-1}$), is highly resistant to heat shock (to 56°C), oxidative stress ($25\text{ mM H}_2\text{O}_2$), and ethanol stress (20%, v/v, ethanol) during growth, and does not gain additional starvation-acquired resistance to either H_2O_2 exposure or heat shock above that already displayed by growing cells. Starved cells do gain additional resistance to ethanol during starvation. Thus the response of *Sphingomonas* sp. strain RB2256 to starvation appears to be fundamentally different from that displayed by some copiotrophs.

Here we report on the starvation-stress survival of *V. anguillarum*, a major marine fish pathogen, which, we suggest, displays a response to starvation that has features different from those described above. This bacterium has been shown to survive prolonged periods of starvation-induced stress (Hoff, 1989). We have examined the starvation-stress responses of *V. anguillarum* with regard to the limiting nutrient, morphological changes during starvation and induction of stress cross-protection. It was found that the starvation-stress response of *V. anguillarum* differs from the responses exhibited by *E. coli*, *P. putida* and *Vibrio* sp. strain S14. First, *V. anguillarum* does not appear to form viable ultramicrocells as the end-point of starvation; instead, surviving cells appear to elongate into helical filaments. Second, stress cross-protection is not uniformly induced and appears slowly. Third, this cross-protection is transient. Cross-protection develops over a 24–72 h period after the initiation of starvation and then, unexpectedly, declines back to the basal, vegetative level. There is a very limited acquisition of thermal tolerance that develops during starvation. In contrast, tolerance to either H_2O_2 , which develops over the first 24 h of starvation, or ethanol, which reaches maximum levels after 72 h of starvation, each results in increased cell survival by as much as several orders of magnitude followed by a decline to near the basal vegetative level. While we do not know why *V. anguillarum* cells are so extremely sensitive to ethanol shock at the initiation of starvation, we speculate that a protective enzyme may be washed out of the periplasm or off the outer

membrane when the cells are prepared for starvation. It should be noted that cells were washed in NSS, which is the base of 3M broth, and were not subjected to an osmotic shock. Additionally, Eguchi *et al.* (1996) have reported a similar sensitivity to ethanol in washed cells of *Sphingomonas* sp. strain RB2256. As reported here for *V. anguillarum*, starved RB2256 cells rapidly regain resistance to ethanol during the first 24 h of starvation. Eguchi *et al.* (1996) have suggested that excreted extracellular metabolites in the culture medium are responsible for ethanol resistance.

At this stage, we cannot definitively explain why *V. anguillarum* cells elongate to form helical cells during starvation. One possibility might be that a FtsZ-like protein is inhibited in starved cells in a manner similar to that observed during the SOS response (Donachie & Robinson, 1987). However, it is interesting to note that the appearance of helical cells during starvation and the loss of starvation-induced cross-protection coincide. We speculate that these two events are linked and may represent a physiological change in the starved *V. anguillarum* cells from a transient stage of relative metabolic shut-down, similar to established starvation-survival responses in other non-differentiating bacteria, to a growth-like but not necessarily replicating state. Additionally, we have observed that the rate of [^{35}S] methionine incorporation into protein on a c.f.u. basis declines about fivefold over the first 6–24 h of starvation and then increases back to levels approaching the rates observed in exponential-phase cells by 7 d of starvation (see Table 1). These observations suggest that the initial response to starvation by *V. anguillarum* is a transient period of decreased metabolic activity which is followed by an active starvation response.

We also note that the c.f.u. in *V. anguillarum* cells starved for phosphorus oscillate with a periodicity of about 300–400 h. Hoff (1989) observed oscillations with a periodicity of about 8 weeks ($\sim 1350\text{ h}$) in the c.f.u. of *V. salmonicida* starved in aged sterile sea water. Hoff could not determine a reason for these oscillations. He did not observe such oscillations in a *V. anguillarum* culture also starved in aged sterile sea water. We hypothesize that the oscillations that we observe may be due to the cycling of phosphorus in our cultures. The c.f.u. values in cultures starved for nitrogen or carbon or multiple nutrients do not oscillate.

One question that remains open is the role of starvation-stress responses in the pathogenicity of *V. anguillarum*. Clearly, both the data reported here and those published previously by Hoff (1989) demonstrate that this pathogen is able to persist in the environment for long periods of time. However, it is not known whether or how the virulence of *V. anguillarum* may be affected by starvation. Future studies will address this problem.

ACKNOWLEDGEMENTS

We thank Paul Johnson of the URI Electron Microscope Facility for providing excellent electron microscopic and photographic services. This investigation was supported by

grants from the USDA (grant no. 93-372-079416) to D. R. N., Kinki University (Grant number 9648) to M. E. and the Australian Research Council to S. K.

REFERENCES

- Austin, B. & Austin, D. A. (1993). *Bacterial Fish Pathogens: Diseases in Farmed and Wild Fish*, 2nd edn. Chichester: Ellis Horwood.
- Bolinches, J., Toranzo, A. E., Silva, A. & Barja, J. L. (1986). Vibriosis as the main causative factor of heavy mortalities in the oyster culture industry in northwestern Spain. *Bull Eur Assoc Fish Pathol* 6, 1–4.
- Bowser, P. R., Rosemark, R. & Reiner, C. (1981). A preliminary report of vibriosis in cultured American lobster, *Homarus americanus*. *J Invertebr Pathol* 37, 80–85.
- Donachie, W. D. & Robinson, A. C. (1987). Cell division: parameter values and the process. In *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology*, vol. 2, pp. 1578–1593. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- Eguchi, M., Nishikawa, T., MacDonald, K., Cavicchioli, R., Gottschal, J. C. & Kjelleberg, S. (1996). Responses to stress and nutrient availability by the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256. *Appl Environ Microbiol* 62, 1287–1294.
- Givskov, M., Eberl, L. & Molin, S. (1994a). Responses to nutrient starvation in *Pseudomonas putida* KT2442: two dimensional electrophoretic analysis of starvation- and stress-induced proteins. *J Bacteriol* 176, 4816–4824.
- Givskov, M., Eberl, L., Møller, S., Poulsen, L. K. & Molin, S. (1994b). Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. *J Bacteriol* 176, 7–14.
- Hengge-Aronis, R. (1993). The role of *rpoS* in early stationary-phase gene regulation in *Escherichia coli* K12. In *Starvation in Bacteria*, pp. 171–200. Edited by S. Kjelleberg. New York: Plenum Press.
- Hoff, K. A. (1989). Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. *Appl Environ Microbiol* 55, 1775–1786.
- Holmquist, L. & Kjelleberg, S. (1993). Changes in viability, respiratory activity and morphology of the marine *Vibrio* sp. strain S14 during starvation of individual nutrients and subsequent recovery. *FEMS Microbiol Ecol* 12, 215–224.
- Jenkins, D. E., Schultz, J. E. & Matin, A. (1988). Starvation induced cross protection against heat of H₂O₂ challenge in *Escherichia coli*. *J Bacteriol* 170, 3910–3914.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Marden, P., Tunlid, A., Malmcrona-Friberg, K., Oldham, G. & Kjelleberg, S. (1985). Physiological and morphological changes during short term starvation of marine bacterial isolates. *Arch Microbiol* 142, 326–332.
- Marouga, R. & Kjelleberg, S. (1996). Synthesis of immediate upshift (Iup) proteins during recovery of marine *Vibrio* sp. strain S14 subjected to long-term carbon starvation. *J Bacteriol* 178, 817–822.
- Matin, A. (1991). The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. *Mol Microbiol* 5, 3–10.
- Matin, A., Auger, E. A., Blum, P. H. & Schultz, J. E. (1989). Genetic basis of starvation survival in non-differentiating bacteria. *Annu Rev Microbiol* 43, 293–316.
- Moriarty, D. J. W. & Bell, R. T. (1993). Bacterial growth and starvation in aquatic environments. In *Starvation in Bacteria*, pp. 25–53. Edited by S. Kjelleberg. New York: Plenum Press.
- Morita, R. Y. (1993). Bioavailability of energy and the starvation state. In *Starvation in Bacteria*, pp. 1–23. Edited by S. Kjelleberg. New York: Plenum Press.
- Neidhardt, F., Block, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* 119, 736–747.
- Nelson, D. R. & Killeen, K. P. (1986). Heat shock proteins of vegetative and fruiting *Myxococcus xanthus* cells. *J Bacteriol* 168, 1100–1106.
- Nelson, D. R. & Zusman, D. R. (1983). Evidence for long-lived mRNA during fruiting body formation in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* 80, 1467–1471.
- Nyström, T., Albertson, N. H., Flärdh, K. & Kjelleberg, S. (1990). Physiological and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp. S14. *FEMS Microbiol Ecol* 74, 129–140.
- Nyström, T. & Kjelleberg, S. (1987). The effect of cadmium on starved heterotrophic bacteria isolated from marine waters. *FEMS Microbiol Ecol* 45, 143–153.
- Östling, J., Holmquist, L., Flärdh, K., Svenblad, B., Jouper-Jaan, Å. & Kjelleberg, S. (1993). Starvation and recovery of *Vibrio*. In *Starvation in Bacteria*, pp. 103–127. Edited by S. Kjelleberg. New York: Plenum Press.
- Poindexter, J. S. (1981). Oligotrophy: fast and famine existence. *Adv Microb Ecol* 5, 63–89.
- Rozak, D. B. & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiol Rev* 51, 365–379.
- Schut, F., De Vries, E. J., Gottschal, J. C., Robertson, B. R., Harder, W., Prins, R. A. & Button, D. K. (1993). Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. *Appl Environ Microbiol* 59, 2150–2160.
- Sonenshein, A. L. (1989). Metabolic regulation of sporulation and other stationary-phase phenomena. In *Regulation of Prokaryotic Development: Structural and Functional Analysis of Bacterial Sporulation and Germination*, pp. 109–130. Edited by I. Smith, R. A. Slepecky & P. Setlow. Washington, DC: American Society for Microbiology.
- Spector, M. P. & Foster, J. W. (1993). Starvation-stress response (SSR) of *Salmonella typhimurium*. Gene expression and survival during nutrient starvation. In *Starvation in Bacteria*, pp. 201–224. Edited by S. Kjelleberg. New York: Plenum Press.
- Zusman, D. R. (1984). Developmental program of *Myxococcus xanthus*. In *Development and Cell Interactions*, pp. 185–213. Edited by E. Rosenberg. New York: Springer.

Received 23 September 1996; revised 11 February 1997; accepted 7 March 1997.