The carbon starvation stimulon in the marine *Vibrio* sp. S14 (CCUG 15956) includes three periplasmic space protein responders

LOUISE HOLMQUIST and STAFFAN KJELLEBERG*

Department of General and Marine Microbiology, University of Göteborg, Carl Skottbergs Gata 22, S-413 19 Göteborg, Sweden

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Growth arrest and long-term starvation in the marine *Vibrio* sp. S14 induce alterations of the cell envelope as well as a sequential expression of starvation-specific polypeptides. The induction and accumulation of three dominant carbon-starvation-induced periplasmic space protein responders of molecular mass 120 (Csp1), 37 (Csp5) and 30 kDa (Csp6), were examined under different starvation and stress conditions. All three proteins were increasingly synthesized in response to carbon and multiple-nutrient starvation, but not during nitrogen and phosphorus starvation, nor in response to the imposition of stress conditions. Csp1 exhibited a more than 100-fold increased relative induction by carbon and multiple-nutrient starvation and was continuously synthesized throughout the experiment. Induction of Csp1 was completely repressed by all conditions other than carbon starvation. A less than fivefold increase in induction was monitored for the transient responders Csp5 and Csp6 within the first few hours of carbon starvation. Interestingly, Csp6 exhibited an increased transient induction at the onset as well as after 24 h of starvation. Accumulation of the three proteins was monitored by Western blot analysis using specific antisera. Of the conditions tested, long-term accumulation of these proteins was only detected when *Vibrio* sp. S14 cells were exposed to carbon and multiple-nutrient starvation. These results are discussed in relation to the recent finding that formation of starvation- and stress-resistant marine *Vibrio* is induced by carbon but not nitrogen or phosphorus starvation, and that Csp1, Csp5 and Csp6 will allow detailed studies of the carbon starvation stimulon.

Introduction

Nearly all ecosystems are oligotrophic, which means that any micro-organism, indigenous or introduced, must face periods with a shortage of energy. To survive under substrate-limiting conditions in marine waters some non-differentiating bacteria respond to starvation by the formation of ultramicrocells, and when appropriate and sufficient nutrients are available they immediately start to grow and divide (Morita, 1988, 1990).

The marine non-differentiating heterotrophic *Vibrio* sp. S14 undergoes a series of morphological and physico-chemical changes when exposed to starvation conditions. Several of these changes have been reported to occur in the cell envelope. For example, an enlarged periplasmic space during starvation, probably caused by an uneven shrinkage of the cytoplasmic membrane relative to the outer membrane, has been shown both for *Vibrio* sp. S14 (Mårdén *et al.*, 1985) and *Escherichia coli* (Reeve *et al.*,

1984). It has also been shown that *Vibrio* sp. S14 switches from low- to high-affinity uptake systems both for amino acids and sugars (Albertson *et al.*, 1990c; Mårdén *et al.*, 1987), similar to responses observed for *Vibrio* sp. ANT-300 (Geesey & Morita, 1979). Furthermore, starved *Vibrio* sp. S14 cells show an increased positive chemotaxis towards some amino acids and sugars during the initial few hours of starvation (Malmcrona-Friberg *et al.*, 1990). Many of these alterations are dependent on *de novo* protein synthesis. Given the starvation-induced alterations in the periplasmic space characteristics, it is likely that this compartment may play a significant role in the physiology of stationary phase and starved cells.

De novo protein synthesis under starvation conditions has been intensively studied in the organisms *Vibrio* sp. ANT-300 (Amy & Morita, 1983), *E. coli* (Groat *et al.*, 1986), *Salmonella typhimurium* (Spector *et al.*, 1986) and *Vibrio* sp. S14 (Nyström *et al.*, 1988, 1990, 1992). These studies have generally focused on the global responses of the cell rather than the localization of the individual protein responders. Furthermore, the assumption that the successful development of starvation- and stress-resistant cells of non-differentiating bacteria occurs

* Author for correspondence. Tel. 46 31 41 87 00; fax 46 31 82 67 90.

Abbreviation: SHMT, serine hydroxamate.

independent of whether the cells are starved for carbon, nitrogen or phosphorus has focused the interest on general starvation proteins. It was demonstrated recently, however, that carbon rather than nitrogen or phosphorus starvation is the determinant for ultra-microcell formation. Hence, information on the carbon starvation stimulon and its specific member genes and proteins is needed. The present study is the first attempt to characterize such carbon-starvation-specific proteins in *Vibrio*. Three such specific responders in *Vibrio* sp. S14 were found to be located in the periplasm. The carbon-starvation-induced periplasmic space proteins, Csp1 (120 kDa) (Albertson *et al.*, 1987), Csp5 and Csp6, were studied at the level of induction and accumulation.

Characterization of the global regulatory networks, induced in starved cells, and their overlap provide further information on the starvation-induced adaptation programme. In the present study we report whether induction of Csp1, Csp5 and Csp6 occurs by exposure to the following stress conditions; heat shock, cold shock, oxidative stress, serine hydroxamate (SHMT) or CdCl₂ addition, anaerobiosis and ethanol exposure. In addition, to test whether the polypeptides were induced also by catabolite repression, cAMP was added.

Methods

Organism, cultivation, starvation and stress conditions. The marine *Vibrio* sp. S14 (CCUG 15956) used in this study has been described previously (Albertson *et al.*, 1990a; Mårdén *et al.*, 1987). Based on biochemical determinations and 16S rRNA sequencing (S. Zabkar and others, unpublished data), it belongs to the core of the genus *Vibrio*. *Vibrio* sp. S14 cells were grown at 24 °C on a rotary shaker in a marine minimal medium (MMM) (Östling *et al.*, 1991) supplemented with 0.4% glucose. *Vibrio* sp. S14 has a growth rate of 0.9 doublings h⁻¹ under the conditions described. Cells were harvested in the exponential phase (2.0×10^8 cells ml⁻¹, OD₆₁₀ = 0.2) by centrifugation (8000 g, 10 min), washed twice and resuspended in the appropriate starvation media. Starvation regimes were kept at 24 °C on slow rotation.

Different nutrient limitations were achieved by resuspending the growing cells in growth medium lacking glucose, phosphorus, nitrogen, or all three nutrients, i.e. multiple-nutrient starvation. For the other stress conditions, aliquots of the exponentially growing cells were exposed to SHMT (10 mM), CdCl₂ (500 µM), ethanol (10%), H₂O₂ (60 µM), heat shock (shift from 24 to 40 °C), cold shock (shift from 24 to 5 °C) and anaerobiosis (N₂ flush). cAMP was added at a concentration of 5 mM (Nyström *et al.*, 1990).

Viability. Viability was measured by c.f.u. on VNSS agar (Vääänen, 1976) using the drop plate method (Hoben & Somasegaran, 1982).

Pulse-labelling experiments, periplasmic space and whole cell protein preparation. During exposure for various starvation conditions, 15 ml of the cell suspension was labelled for 10 min during 0, 0.5, 3 and 8 h starvation with 50 µCi [³⁵S]methionine (Dupont, New England Nuclear; 1 Ci = 37 GBq). For the longer starvation periods (24, 48 and 96 h) the pulse lasted 20 min. Pulse-labelling of stress-induced cells was made subsequent to a 10 min prestress period for the individual stress inducer. The cells were labelled for a period of 10 min, except for the cold shock experiment where the cells had been chilled to 5 °C for

50 min in a refrigerator before labelling. After labelling, cells were filtered onto 0.22 µm membrane filters (Poretics), washed with the appropriate medium and resuspended in 1 ml sterile distilled water and incubated for 15 min. This allowed for osmotic shock release of the periplasmic space contents (Mårdén *et al.*, 1987). Cells were centrifuged at 15000 g in a microfuge (Beckman). The supernatant, which contained the periplasmic space proteins, was filter sterilized (0.22 µm) and lyophilized. [This method is highly reproducible and the osmotically shocked cells remain 100% viable. Furthermore, comparisons of the periplasmic fraction, and the cytoplasmic and membrane fractions by O'Farrell two-dimensional gel electrophoresis were performed (data not shown). Csp1, Csp5 and Csp6 cannot be found in the latter fractions.] A 50 µl filtrate subsample was withdrawn before lyophilization to measure the amount of radioactivity incorporated into polypeptides by precipitation with 5% (w/v) cold TCA. The precipitates were collected on membrane filters and resuspended in scintillation cocktail (Aquasure, NEN Dupont), and the radioactivity counts were determined using a scintillation counter (Beckman model LS 6000 LL). Extraction of whole cell protein was done by precipitation with TCA and the precipitation was processed as described above for determining radioactivity.

Gel electrophoresis. The periplasmic fractions were resuspended in 50 µl 10 mM-Tris/HCl pH 8.0 and 10 µl SDS-PAGE sample buffer and analysed by electrophoresis on 12% polyacrylamide gels using the Laemmli buffer system (LKB, Pharmacia). Fifty µg protein containing a known radioactivity was loaded on the gels. The separated proteins were transferred to nitrocellulose (Schleicher & Schuell) by electroblotting using Mini-Protean and Mini-Transblot apparatus (BioRad), following the manufacturer's instructions. The electroblotted gels were stained with Coomassie brilliant blue G250 to check for 100% transfer. The membranes were subjected to autoradiography overnight and Western blot analysis was performed using the same membranes. Autoradiograms were scanned using a LKB Ultrosan XL 2220 densitometer at 633 nm. The relative induction was calculated by dividing the absorbance of each band by the total radioactivity and length of the pulse loaded on the gel. Similarly, the relative amount and accumulation of the proteins were calculated by dividing the absorbance of each band by the protein concentration loaded on the gel.

Protein concentration measurements. An aliquot from each resuspended periplasmic fraction was used to measure the protein concentration. Twenty microlitres was analysed using the Pierce BCA microtitre assay protocol at 37 °C. Bovine serum albumin (BSA) was used as standard. Protein concentrations were determined by using a microtitre plate reader at 545 nm (Biotek Instruments model EL312e).

Antisera preparation and Western blot analysis. Ten preparative SDS-PAGE gels (1.5 mm thick) were run with periplasmic fractions from *Vibrio* sp. S14 cells that had been starved for carbon for 24 h, using the Laemmli buffer system. The gels were stained with Coomassie brilliant blue G250 and the bands corresponding to Csp5 and Csp6 were cut out using a razor blade. The gel pieces were homogenized by sequentially squeezing through thinner gauge needles, the finest of which was 18 gauge. The homogenates of Csp5 and Csp6 so obtained were injected intradermally (50 mg per injection) into two New Zealand white female rabbits four times, with 2 week interval between each injection. Freund's complete adjuvant was used for the first injection; thereafter we used Freund's incomplete adjuvant. Each antiserum obtained was used at a dilution of 1:2000. The antibody recognizing Csp1 was prepared by Albertson *et al.* (1987). For Western blotting we used the method recommended by the manufacturer (Stratagene, Picoblu immunoscreening kit), except that we used 2% (v/v) Tween-20 instead of BSA for blocking of background non-specific sites. An alkaline phosphatase-conjugated goat anti-rabbit antibody was used as the secondary antibody.

Results

Incorporation of radiolabelled methionine in the periplasmic space proteins during starvation

Changes in the degree of incorporation of labelled methionine during different starvation regimes and at different times of starvation were measured by pulse-labelling starved *Vibrio* sp. S14 cells (Fig. 1). Multiple-nutrient starvation showed a less than threefold increase in the relative rate of incorporation, but it did not exhibit the secondary increase displayed by carbon-starved cells. Phosphorus starvation induced an immediate drop in protein synthesis prior to a return to the synthesis observed during growth conditions after 24 h of starvation.

Fig. 1. Relative incorporation of radiolabelled methionine into the periplasmic space proteins of *Vibrio* sp. S14 during starvation $\pm$ SD. Exponentially growing cells were assigned a value of 1.0. Starved cells were pulse-labelled, the periplasmic space proteins were precipitated and radioactive counts were determined in a liquid scintillator. The cells were exposed to either multiple-nutrient (○), carbon (●), nitrogen (■) or phosphorus (□) starvation. The arrow indicates the onset of starvation. The experiment was repeated four times to confirm reproducibility.

vation. Nitrogen and phosphorus starvation gave a similar response. The amount of radioactivity incorporated into the periplasmic space proteins as a percentage of that incorporated into whole cell protein varied with different individual nutrients and times of starvation, increasing from 15 to 40% during the first 8 h of carbon starvation and stabilizing at 20% of total radioactivity in periplasmic space proteins after prolonged starvation. Multiple-nutrient-starved cells exhibited a maximum of 30% at 8 h, whereas nitrogen and phosphorus starvation gave an increase from 15 to 25% during the first 8 h and a decrease to 10% during prolonged starvation. The fact that there were essentially no changes in the protein profile (Fig. 2) indicates that cytoplasmic proteins are not present as contaminants in the periplasmic space fraction, released by osmotic shock, at any of the starvation times tested.

Relative induction of Csp1, Csp5 and Csp6 during starvation for different nutrients

The relative induction of the periplasmic space proteins Csp1, Csp5 and Csp6 was measured by scanning the autoradiograms from the SDS-polyacrylamide gels which were loaded with the pulse-labelled periplasmic space proteins. The presence of a single protein per band was validated by O'Farrell two-dimensional gel electrophoresis (data not shown). Csp1 was synthesized during carbon and multiple-nutrient starvation but was not detected during nitrogen or phosphorus starvation (Fig. 3a). It is noteworthy that the relative induction of Csp1 increased more than 100-fold within 24 h of carbon or multiple-nutrient-starvation. The pattern of induction for both Csp5 and Csp6 was similar in that starvation for nitrogen and phosphorus decreased protein synthesis,

Fig. 2. A non-quantitative autoradiogram of a 12% SDS-polyacrylamide gel of periplasmic space proteins of *Vibrio* sp. S14 cells starved of carbon. The periplasmic space proteins Csp1, Csp5 and Csp6 are indicated. Lanes: 1, exponentially growing cells; 2, 0.5 h; 3, 3 h; 4, 8 h; 5, 24 h; 6, 48 h; and 7, 96 h starvation. Standard molecular mass proteins are indicated. All lanes except lane 1 are from the same gel. Different exposure times were employed for the different lanes for better visualization. The experiment was repeated four times to confirm reproducibility; a representative result is shown.

Fig. 3. Relative induction of the periplasmic space proteins Csp1 (a), Csp5 (b) and Csp6 (c) in *Vibrio* sp. S14 during starvation $\pm$ SD. Exponentially growing cells were assigned a value of 1.0. Starved cells were pulse-labelled and the periplasmic space proteins were separated by SDS-PAGE. Calculations were made from autoradiograms and scanning data (see Methods). The cells were exposed to either multiple-nutrient ($\circ$), carbon ($\bullet$), nitrogen ($\blacksquare$) or phosphorus ($\square$) starvation. The arrow indicates the onset of starvation. Synthesis of Csp1 during nitrogen and phosphorus starvation was not detected. The experiment was repeated four times to confirm reproducibility.

whereas starvation for multiple-nutrients and carbon increased protein synthesis (Figs 3b and c). In contrast, carbon depletion induced synthesis of Csp5 in a manner similar to multiple-nutrient starvation (Fig. 3b), whereas multiple-nutrient starvation was found to be the dominant inducer for synthesis of Csp6 (Fig. 3c). Furthermore, there was a transient decrease in induction of Csp6 by 8 h of carbon starvation. The pattern of synthesis of Csp5 showed strong similarity with the pattern of synthesis of the pool of the periplasmic space proteins (Figs 1 and 3b). The molecular masses of Csp5 and Csp6 were found to be 37 and 30 kDa, respectively. A non-quantitative autoradiogram from a 12% SDS-polyacrylamide gel (Fig. 2) of a carbon starvation time

Table 1. Relative survival and synthesis of Csp5 and Csp6 in *Vibrio* sp. S14 during exposure to different stress conditions

Cells were pulse-labelled and the periplasmic space proteins were separated by SDS-PAGE. Data were calculated from scanned autoradiograms (see Methods). The relative degree of survival was measured by c.f.u. on VNSS agar. Data are given as relative values $\pm$ SD, where untreated exponentially growing cells were assigned a value of 1.0. The experiment was repeated four times to confirm reproducibility.

Stress exposure	Relative survival of cells	Relative synthesis	
		Csp5	Csp6
5 °C	0.62 $\pm$ 0.04	0.87 $\pm$ 0.05	1.04 $\pm$ 0.04
40 °C	0.40 $\pm$ 0.03	0.78 $\pm$ 0.07	1.08 $\pm$ 0.03
N ₂	0.98 $\pm$ 0.01	0.56 $\pm$ 0.02	0.45 $\pm$ 0.01
CdCl ₂	0.72 $\pm$ 0.03	0.38 $\pm$ 0.03	0.54 $\pm$ 0.01
SHMT	0.96 $\pm$ 0.02	0.68 $\pm$ 0.06	0.72 $\pm$ 0.02
Ethanol	0.54 $\pm$ 0.04	0.23 $\pm$ 0.04	0.34 $\pm$ 0.02
cAMP	0.86 $\pm$ 0.06	0.74 $\pm$ 0.05	0.88 $\pm$ 0.02
H ₂ O ₂	0.20 $\pm$ 0.03	0.00	0.00

course shows the induction of the specific proteins in the periplasmic fraction of *Vibrio* sp. S14.

Survival of *Vibrio* sp. S14 and synthesis of Csp1, Csp5 and Csp6 under different stress conditions

To investigate whether the induction of Csp1, Csp5 or Csp6 by exposure to other stress conditions overlaps with carbon and multiple-nutrient starvation, we challenged exponentially growing cells with ethanol, CdCl₂, heat shock, cold shock, anaerobic conditions (N₂) and oxidative stress (H₂O₂), as well as with added SHMT and cAMP. The cells were tested for viability (c.f.u.) immediately after the stress exposure was terminated, and periplasmic space proteins were prepared and analysed by gel electrophoresis (Table 1). The largest decrease in survival was observed when the cells were exposed to heat and oxidative stress: 40 and 20% of the original number of cells remained viable, respectively. No synthesis of Csp1 was detected for any of the stress conditions tested. Except for oxidative stress, synthesis of Csp5 and Csp6 occurred under all stress conditions, but did not exceed the rate of synthesis of the untreated cells.

Detection of Csp1, Csp5 and Csp6 using antibodies during different starvation and stress conditions

Western blotting was performed using the same membranes as those used for autoradiography. This certifies that the band detected by the antibody can be identified on the autoradiogram. Since we loaded equal amounts of protein on the gels, the intensity of the bands on the

Fig. 4. A Western blot showing the periplasmic space protein Csp5 of *Vibrio* sp. S14, detected by anti-Csp5 during a carbon starvation time course experiment. Lanes: 1, exponentially growing cells; 2, 0.5 h; 3, 3 h; 4, 8 h; 5, 24 h; 6, 48 h; and 7, 96 h starvation. The experiment was repeated four times to confirm reproducibility; a representative result is shown.

Table 2. Relative accumulation of Csp1, Csp5 and Csp6 in *Vibrio* sp. S14 detected with specific antibodies

Western blot analysis was performed on periplasmic space proteins of cells starved for carbon. Data are presented as relative values $\pm$ SD, where exponentially growing cells were assigned a value of 1.0. The experiment was repeated four times to confirm reproducibility.

Time of starvation (h)	Relative accumulation		
	Csp1	Csp5	Csp6
0.5	1.40 $\pm$ 0.13	4.11 $\pm$ 0.16	3.76 $\pm$ 0.06
3	3.18 $\pm$ 0.02	4.79 $\pm$ 0.01	4.06 $\pm$ 0.08
8	7.56 $\pm$ 0.09	5.41 $\pm$ 0.16	4.64 $\pm$ 0.06
24	8.40 $\pm$ 0.14	6.46 $\pm$ 0.06	5.86 $\pm$ 0.09
48	2.42 $\pm$ 0.12	7.08 $\pm$ 0.12	4.14 $\pm$ 0.09
96	2.28 $\pm$ 0.01	6.36 $\pm$ 0.09	3.87 $\pm$ 0.10

Western blots, given by scanning with a densitometer, allows for comparative studies. Table 2 shows the accumulation of Csp1, Csp5 and Csp6 during carbon starvation, identified by the antibodies raised against the specific proteins. The concentration of Csp1 decreased during prolonged carbon starvation although synthesis continued, indicating increased degradation of the protein. Whilst there was little or no synthesis of Csp5 and Csp6 after 48 h of carbon starvation, the concentration of these proteins was high, probably due to the low rate of degradation of these proteins. The multiple-nutrient starvation experiment showed essentially the same pattern of protein accumulation as did carbon starvation, whereas for nitrogen and phosphorus starvation, as well as for the different stress conditions tested, no accumulation could be detected (data not shown). Fig. 4 shows a typical Western blot, using anti-Csp5, of a carbon starvation time course experiment.

Discussion

It was recently established that the starvation-induced adaptation programme in *Vibrio* sp. S14 is identical, with respect to the macromolecular synthesis pattern, viability

and induction of cross protection against a variety of stress agents, whether induced by multiple-nutrient or carbon starvation (Nyström *et al.*, 1992). Whilst nitrogen and phosphorus starvation induce the same programme as does carbon starvation, and successfully develop starvation- and stress-resistant small starved cells in *E. coli* (Matin, 1991) and *Bacillus subtilis* (Hecker & Völker, 1990; Losick & Youngman, 1984), the response exhibited by *Vibrio* is clearly very different. The present study confirms the finding reported by Nyström *et al.* (1992) that nitrogen and phosphorus starvation give rise to a different response as compared to carbon and multiple-nutrient starvation. Whilst no loss in viability, compared to the onset of starvation, was observed for the latter starvation conditions in the present study, 96 h nitrogen- and phosphorus-starved cells exhibited a > 99.9% loss in c.f.u. (data not shown). The loss of viability when the cells are starved for nitrogen or phosphorus is probably due to the inability of the cells to divide and form colonies, however, rather than cell death (unpublished).

The only differences observed between multiple-nutrient and carbon starvation in this study are those of the time and extent of maximum synthesis of the pool of proteins in the periplasmic space (Fig. 1), the percentage of radiolabelled periplasmic space proteins compared to radiolabelled whole cell protein, and synthesis of the three individual proteins. In view of these findings, it is suggested that an improved understanding of the starvation-induced adaptation response in marine *Vibrio* will be gained by studies of the carbon starvation stimulon and its member responders rather than those proteins that are induced irrespective of carbon, nitrogen or phosphorus starvation. The carbon starvation network may regulate the adaptation programme and development of starvation- and stress-resistant cells.

This study shows that the periplasmic space proteins Csp1, Csp5 and Csp6 are induced by carbon as well as multiple-nutrient starvation, but not by nitrogen and phosphorus depletion. As with the *pex* genes in *E. coli*, which are essential for development of starvation and stress resistance (Matin, 1991), the genes encoding Csp1, Csp5 and Csp6 are cAMP-independent. As for the total protein synthesis of the periplasmic space polypeptides, the initial phase of nitrogen and phosphorus starvation appears to repress rather than induce synthesis of Csp5 and Csp6. Very importantly, the three Csp proteins appear to represent carbon starvation-specific responders that are induced at different times of the adaptation programme. The sequential induction of carbon starvation genes of this programme can therefore be studied. Csp1 exhibits a > 100-fold increase in its rate of synthesis by 8 h of carbon starvation. A three- to fourfold increase in synthesis for Csp5 and Csp6 peaked after 3 h starvation. The latter proteins were transient responders:

by 48 h of carbon and multiple-nutrient starvation the rate of synthesis was as low as that observed at the onset of starvation. Csp1 appears to be synthesized throughout the entire period of starvation and belongs to the subclass of class A protein responders that are permanently induced during prolonged periods of non-growth (Nyström *et al.*, 1990). However, Csp5 and Csp6 remain stable, as shown by Western blot analysis, throughout the starvation period of 96 h employed in this study. Again, the multiple-nutrient and carbon starvation-induced cells showed an almost identical pattern of accumulation, whereas the Western blot analysis did not detect any accumulation of Csp1, Csp5 and Csp6 for nitrogen- or phosphorus-starved cells.

It is in fact feasible that the three periplasmic space proteins examined in this study belong exclusively to the carbon starvation stimulon. In addition to the lack of induction by nitrogen and phosphorus starvation, neither amino acid starvation (SHMT addition), cAMP addition or any of the six stress conditions evoked an increased synthesis of Csp1, Csp5 and Csp6. Further studies of these as well as the regulation of the encoding genes may therefore provide a tool for elucidating the starvation-induced adaptation and formation of small starved cells in marine *Vibrio* strains.

As deduced from the pronounced alterations observed in cell-envelope-related characteristics of starving cells, it is likely that several of the 66 starvation-induced genes detected during the initial few days of multiple-nutrient starvation direct the synthesis of proteins, the destination of which is the periplasmic space. Whilst several studies of starvation-specific protein responders have been reported for *E. coli*, *S. typhimurium* and marine *Vibrio* strains (Amy & Morita, 1983; Groat *et al.*, 1986; Nyström *et al.*, 1988, 1990, 1992; Spector *et al.*, 1986), few attempts have been made to assign these responders to a cellular location. In one such study, Nyström *et al.* (1988) identified starvation-specific proteins in the periplasmic space of *Vibrio* sp. S14 which were sequentially induced over a period of 24 h multiple-nutrient starvation.

Observations on starvation-induced phenotypic alterations have revealed a series of changes that most likely reflect shifts in the protein composition of the periplasmic space of different *Vibrio* strains. The induction of high affinity uptake systems for amino acids and sugars has been described at various times after growth arrest (Albertson *et al.*, 1990c; Geesey & Morita, 1979; Mårdén *et al.*, 1987). The identification of the binding proteins involved in these responses has also been reported. Several studies have demonstrated starvation-specific alterations in chemotaxis towards amino acids and sugars (Malmcrona-Friberg *et al.*, 1990), which possibly involve binding proteins for delivery of the attractant

to the membrane-bound transducer. Additional periplasmic-space-localized starvation proteins are exoproteases. It has been demonstrated that an increased exoprotease activity correlates with the growth-arrest-induced differentiation response in two marine bacterial strains (Albertson *et al.*, 1990a). Such an activity is most likely to stimulate the cell to induce new uptake systems that allow the cell to mobilize substrates otherwise inaccessible to them.

The molecular masses of Csp5 and Csp6 were found to be 37 and 30 kDa, respectively. Interestingly, these are within the size range of most binding proteins of Gram-negative bacteria (22–41 kDa; Philips *et al.*, 1987). We have not yet been able to assign a function to the periplasmic space proteins Csp1, Csp5 and Csp6. N-terminal sequences of these proteins have been made and these do not start with methionine, indicating cleaved signal sequences (data not shown). Homologies with other proteins in available databases have not been found. The gene encoding Csp5 has been cloned and is presently being sequenced (unpublished). This, as well as cloning and sequencing of Csp1 and Csp6, will allow us to identify regulatory regions of carbon-starvation-specific genes that are transcribed at different times of the adaptation programme and generate site-specific mutants in order to further understand the function of carbon-starvation-specific proteins.

It is worth mentioning that one of three carbon-starvation-specific periplasmic space proteins, Csp1, was identified as the product of an mRNA species with a functional half-life of 71 min after 24 h of multiple-nutrient starvation (Albertson *et al.*, 1990b). Antibodies raised against 24-h-starved cells were previously found to specifically detect a limited number of outer membrane and periplasmic space proteins (Albertson *et al.*, 1987), one of which was subsequently identified as the product of the long-lived Csp1 mRNA transcript (Albertson *et al.*, 1990b). The induction of long-lived transcripts may represent one way for the cell to regulate and minimize the cost for synthesis of essential proteins during starvation.

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References

- ALBERTSON, N. H., JONES, G. W. & KJELLEBERG, S. (1987). The detection of starvation-specific antigens in two marine bacteria. *Journal of General Microbiology* **133**, 2225–2231.
- ALBERTSON, N. H., NYSTRÖM, T. & KJELLEBERG, S. (1990a). Exoprotease activity of two marine bacteria during starvation. *Applied and Environmental Microbiology* **56**, 218–223.
- ALBERTSON, N. H., NYSTRÖM, T. & KJELLEBERG, S. (1990b). Functional mRNA half-lives in the marine *Vibrio* sp. S14 during starvation and recovery. *Journal of General Microbiology* **136**, 2195–2199.

- ALBERTSON, N. H., NYSTRÖM, T. & KJELLEBERG, S. (1990c). Starvation-induced modulations in binding protein-dependent glucose transport by the marine *Vibrio* sp. S14. *FEMS Microbiology Letters* **70**, 205–210.
- AMY, P. S. & MORITA, R. (1983). Protein patterns of growing and starved cells of a marine *Vibrio* sp. *Applied and Environmental Microbiology* **45**, 1748–1752.
- GEESEY, G. G. & MORITA, R. Y. (1979). Capture of arginine at low concentrations by a marine psychrophilic bacterium. *Applied and Environmental Microbiology* **38**, 1092–1097.
- GROAT, R. G., SCHULTZ, J. E., ZYCHLINSKY, E., BOCKMAN, A. & MATIN, A. (1986). Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. *Journal of Bacteriology* **168**, 486–493.
- HECKER, M. & VÖLKER, U. (1990). General stress proteins in *Bacillus subtilis*. *FEMS Microbiology Ecology* **74**, 197–213.
- HOBEN, H. J. & SOMASEGARAN, P. (1982). Comparison of the pour, spread and the drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Applied and Environmental Microbiology* **44**, 1246–1247.
- LOSICK, R. & YOUNGMAN, P. (1984). Endospore formation in *Bacillus*. In *Microbial Development*, pp. 63–88. Edited by R. Losick & L. Shapiro. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MALMCRONA-FRIBERG, K., GOODMAN, A. & KJELLEBERG, S. (1990). Chemotactic responses of a marine *Vibrio* sp. strain S14 (CCUG 15956) to low-molecular-weight substances under starvation-survival conditions. *Applied and Environmental Microbiology* **56**, 3699–3704.
- MÄRDÉN, P., NYSTRÖM, T. & KJELLEBERG, S. (1987). Uptake of leucine by a marine Gram-negative heterotrophic bacterium during exposure to starvation conditions. *FEMS Microbiology Ecology* **45**, 233–241.
- MÄRDÉN, P., TUNLID, A., MALMCRONA-FRIBERG, K., ODHAM, G. & KJELLEBERG, S. (1985). Physiological and morphological changes during short term starvation of marine bacterial isolates. *Archives of Microbiology* **142**, 326–332.
- MATIN, A. (1991). The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. *Molecular Microbiology* **5**, 3–10.
- MORITA, R. Y. (1988). Bioavailability of energy and its relationship to growth and starvation survival in nature. *Canadian Journal of Microbiology* **34**, 436–441.
- MORITA, R. Y. (1990). The starvation-survival state of microorganisms in nature and its relationship to the bioavailable energy. *Experientia* **46**, 813–817.
- NYSTRÖM, T., ALBERTSON, N. & KJELLEBERG, S. (1988). Synthesis of membrane and periplasmic proteins during starvation of a marine *Vibrio* sp. *Journal of General Microbiology* **134**, 1645–1651.
- NYSTRÖM, T., FLÄRDH, K. & KJELLEBERG, S. (1990). Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. *Journal of Bacteriology* **172**, 7085–7097.
- NYSTRÖM, T., OLSSON, R. M. & KJELLEBERG, S. (1992). Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. S14 during starvation for different individual nutrients. *Applied and Environmental Microbiology* **58**, 55–65.
- ÖSTLING, J., GOODMAN, A. & KJELLEBERG, S. (1991). Behaviour of IncP-1 plasmids and a miniMu transposon in a marine *Vibrio* sp.: isolation of starvation-inducible *lac* operon fusions. *FEMS Microbiology Ecology* **86**, 83–94.
- PHILIPS, T. A., VAUGHN, V., BLOCH, P. L. & NEIDHARDT, F. C. (1987). Gene protein index of *Escherichia coli* K-12, edition 2. In *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology*, pp. 919–966. Edited by F. C. Neidhardt, J. O. Ingraham, K. Brooks Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, D.C.: American Society for Microbiology.
- REEVE, C. A., AMY, A. S. & MATIN, A. (1984). Role of protein synthesis in the survival of carbon starved *Escherichia coli* K-12. *Journal of Bacteriology* **160**, 1041–1046.
- SPECTOR, M. P., ALIABADI, Z., GONZALEZ, T. & FOSTER, J. W. (1986). Global control in *Salmonella typhimurium*: two-dimensional electrophoretic analysis of starvation-, anaerobiosis-, and heat shock-inducible proteins. *Journal of Bacteriology* **168**, 420–424.
- VÄÄTÄNEN, P. (1976). Microbiological studies in coastal waters of the Northern Baltic sea. I. Distribution and abundance of bacteria and yeasts in the Tvärminne area. *Walter and Andre de Nottbeck Foundation Scientific Report*, **1**, 1–58.