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The Effect of Starvation Stress on the Protein Profiles in *Flexibacter chinensis*

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ABSTRACT

Background: Analysis of many proteins produced during the transition into the stationary phase and under stress conditions (including starvation stress) demonstrated that a number of novel proteins were induced in common to each stress and could be the reason for cross-protection in bacterial cells. It is necessary to investigate the synthesis of these proteins during different stress conditions. **Methods:** The changes in protein profile of *Flexibacter chinensis* at various stages of the starvation process and the other stresses were investigated using two-dimensional gel electrophoresis which has proven to be a powerful tool for investigation of the changes in protein profiles under such conditions. **Results:** Most starvation proteins were synthesized during the early stationary phase and many of these proteins remained during long-term starvation. Some of these proteins were transiently synthesized. The sequencing result of one of the proteins showed that there was a 62.5% identity in 8 amino acids overlapped with the 5' residue of a 10 kDa chaperon protein which is known to be involved in the starvation stress response in other organisms. **Conclusion:** There are many proteins synthesized in common with many stresses in *Flexibacter chinensis*. Some of these proteins must play a major role in the stability of the cell under different stresses. *Iran. Biomed. J. 12 (2): 67-75, 2008*

Keywords: Flexibacter chinensis, Starvation stress, Protein profile

INTRODUCTION

During persistence in the eukaryotic host environment, prokaryotic cells may enter a quiescent state, reminiscent of the stationary phase of laboratory cultures, due to conditions which do not support the rapid bacterial multiplication [1, 2].

Prokaryotic cells respond to environmental or chemical stress by inducing specific sets of proteins characteristic to each stress. The proteins in each set of their coding genes constitute a stimulon, such as heat shock, SOS response and oxidation stress. In some other cases, proteins which associated with one stimulon can be induced during other stresses, such as various heat shock proteins in *E. coli*. These proteins are also synthesized when the cells are exposed to hydrogen peroxide, ethanol, UV light, puromycin or amino acid deprivation. In some stimulons, exposure to non-lethal levels of a stress agent can confer protection against subsequent exposure to lethal levels of the same stress agent [3, 4]. A response regulator in *Mycobacterium smegmatis* which plays an important role in adaptation to oxygen-starved stationary phase was reported by Ronan *et al.* [5] and a protein which is vital for surviving of *Pseudomonas aeruginosa* was also reported [6]. This protein also is essential for anaerobic condition growth in *Pseudomonas aeroginosa* [7]. In stationary phase, the formation of non-culturable but viable cells depends on production of special proteins [8, 9].

Forty starvation-related proteins which also confer enhanced resistance to heat, oxidation stress, antibiotics and proteolysis are induced in the stationary phase in *E. coli*. Starvation-survival depends on novel protein synthesis [10] and a set of novel proteins are produced during stationary phase in *Mycobacterium smegmatis* [11]. A subset of starvation proteins, pex proteins, plays a significant

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role in the resistance to starvation. They are synthesized under carbon, nitrogen or phosphorus and multiple nutrient starvation and, in addition, some pex proteins are induced during heat or oxidation stress [10]. Although starvation for individual nutrients and other stresses induces a unique and individual profile of protein expression, some proteins are common to different starvation and stress factors. However, the proteins of one stimulon do not respond coordinately to all starvation and stress treatments and relatively few of the starvation-inducible proteins have been found to overlap with those induced by stress [12]. This suggests that, despite the regulation of a few specific proteins being interconnected, there are major differences in the regulatory pathways controlling the expression of starvation and different stress proteins [12]. In *E. coli*, several starvation proteins are common to heat shock, a condition under which protein induction is controlled by the minor sigma factor δ^{32}. Therefore, this sigma factor must play a role in the induction of proteins during both heat shock and starvation stress [10].

In this research, the *Flexibacter chinensis* was selected due to long-term starvation and stress toleration compared to *E. coli*. Regarding to our previous works on several stress affects on *Flexibacter chinensis* cell survival, it is demonstrated that this organism remains viable for a period of long time during starvation and stress conditions. These data suggest that using multi-microorganisms may be a better pattern in water pollution. In this study, the changes to the protein profiles from cells exposed to a variety of stresses were examined. These profiles were observed using two-dimensional gel electrophoresis.

MATERIALS AND METHODS

Bacterial strain. The bacterial strain used in this study was *Flexibacter chinensis* obtained from Ken Flint, Warwick University (UK).

Bacterial growth media and conditions. The Flexibacter strains were routinely grown in Luria broth (10 g/l Bacto tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.2) or on Luria Agar (10 g/l bacto tryptone, 5 g/l yeast extract, 5g/l NaCl, 15 g/l Agar). Plates were incubated at 30°C.

Starvation protocol. *F. chinensis* was grown in Luria broth at 30°C for 24 h (5,000 ×g). The Culture (10 ml) was centrifuged and washed twice using sterile distilled water and the pellet was resuspended in final volume of 10 ml of sterile distilled water. Resuspended culture (0.1 ml) was inoculated into 100 ml of sterile water in a 250- ml Erlenmeyer flask to give an initial viable count of around 10^7 colony forming units/ml (cfu/ml).

Sample preparation for two-dimensional gel electrophoresis. *F. chinensis* was grown in a suitable liquid medium at 30°C for 24 h. The cells were harvested by centrifugation and sonicated in 1 mM Tris buffer (pH 8.0). The sonicated cells were centrifuged at 1,500 ×g for 20 min at 4°C. The pellet was discarded and the supernatant centrifuged at 48,400 ×g for 60 min at 4°C to harvest the membrane proteins and leave the soluble proteins in the supernatant. The membrane proteins were resuspended in 3 ml Tris buffer (pH 8.0) and stored at -20°C. The supernatant was concentrated using an Amicon ultra-filtration apparatus to about 5 ml. An equal amount of saturated ammonium sulphate was added to the concentrated supernatant fraction to give a final ammonium sulphate concentration of 50%, and left overnight to allow the proteins to precipitate. The precipitate was pelleted by centrifugation at 35,000 ×g for 30 min, resuspended in 5 ml of sterile distilled water and dialyzed overnight against at least 4 liter of distilled water to remove any ammonium sulphate from the samples [13].

Two-dimensional gel electrophoresis. This technique was done according to the method described by O'Farrell [14]. In this technique, the range of pH was selected between 3-10 in the first dimensional phase and the SDS-PAGE gel was prepared 12% in the second dimensional phase. The gel was stained with silver nitrate and the samples for 2DE were repeated for three times. Images of 2D gels are acquired into a database using an image scanner. Image analysis software converts the gel image into a digitized image in a computer, matches gels and spots on gels across the different groups and creates a database with information about spot intensity and spot location.

N- terminal amino acid sequencing of proteins. The proteins, identified as being specific for starvation by two-dimensional gel electrophoresis, were selected for transfer to a membrane for sequencing. The gel was incubated in a low glycine transfer buffer (methanol, 100 ml; Tris, 5.81g;

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glycine, 2.93 g; SDS, 0.3 g; water, 900 ml) for 15 min. Hydrophobic polyvinylidene difluoride (PVDF) membrane from Bio-Rad (USA) was soaked in 100% (v/v) methanol and transferred into the glycine transfer buffer for 15 min. The proteins were blotted on to the PVDF membrane by placing a piece of PVDF membrane cut to the same size as the gel on top of the gel. The gel and filter were placed between six shots of filter paper and soaked in the low glycine transfer buffer (100 ml methanol, 5.81 g Tris, 2.93 g Glycine, 0.3 g SDS and 900 ml water) for 15 minutes. A porous sponge was placed on each side of the filter paper sandwich and the hole was put into the cassette of a trans blot tank containing the low glycine transfer buffer. The protein was transferred into the PVDF membrane at 65 volt at 4°C for 3 h and stained with Coomassie Blue (0.025% (w/v) Coomassie Blue R250 (Sigma, USA) in 40% (v/v) methanol for 5 min and destained in 50% (v/v) methanol until the protein spots were visible. Selected spots were cut out and send to Leicester University (Leicester, UK) for N-terminal sequencing.

RESULTS

Protein profile of F. chinensis in the exponential, early stationary phase and late stationary phase of growth. In these experiments, the cells were routinely grown at 15°C for the optimal growth temperature for *F. chinensis* which would not produce any effects due to heat shock.

A number of changes occurred in the protein profiles during the transition of the cells from exponential phase to early stationary phase. Proteins (n = 28) were newly synthesized and most of them were still present in the cells later in the stationary phase. However, more than 10 proteins disappeared or their syntheses were repressed during this transition (Fig. 1a and 1b).

Figures 1c and 1d compare the changes in the protein profiles during the transition from early stationary phase to mid stationary phase. Figures 1b and 1c are of the same gel but the proteins which change are different and indicated as such. Twenty nine new proteins were induced that a few of them seem to be only produced in this period, as they are absent from cells in the early stationary phase and long-term starved cells. The synthesis of four proteins was repressed in this phase as judged by the number of proteins disappeared between two phases.

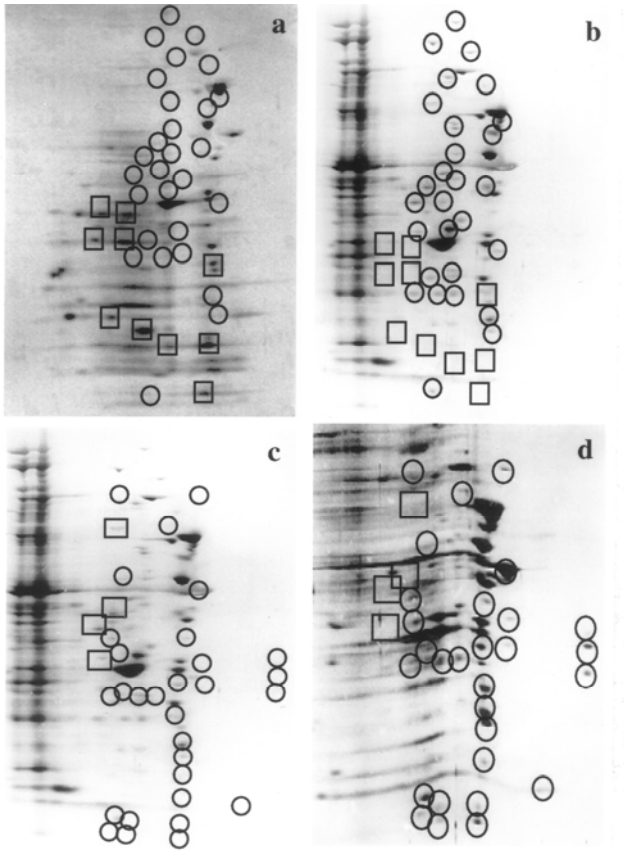


Fig. 1. Two-dimensional electrophoresis analysis. The Figure shows the protein profile **(a)** for exponential phase cells (6 h), **(b** and **c)** for early stationary phase cells (10 h) and **(d)** for mid stationary phase cells (18 h). Protein profiles were compared and proteins which disappeared **(□)** and appeared **(**O**)** are indicated.

Figure 2 shows the comparison between the protein profiles of early and mid stationary phase compared with the late stationary phase. Comparison of Figures 2a and 2b shows that 11 new proteins were induced and 18 proteins disappeared or their synthesis was repressed during the mid stationary phase to the late stationary phase. Figures 2c and 2d compare the protein profiles of early stationary phase cells with those in the late stationary phase. The number of newly synthesized proteins was 27 and the number of proteins which disappeared was 7. These are the same gels described in Figure 1 but the changes are indicated in the Figure 2 for clarity.

Comparison of the protein profiled of F. chinensis in early, mid and late stationary phase with multiple starvation stress. In this experiment (Fig. 3) the protein profiled of the cells subjected to starvation stress for all nutrients for up to 7 days was

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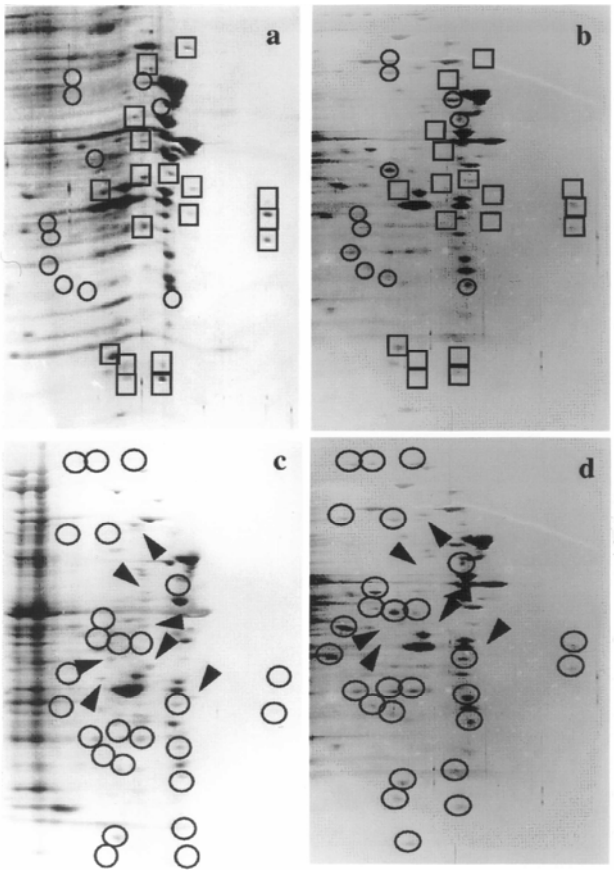


Fig. 2. Two-dimensional electrophoresis analysis**.** The Figure shows the protein profile **(a)** for mid stationary phase cells, **(b** and **d)** for late stationary phase cells and **(c)** for early stationary phase cells. Protein profiles were compared and proteins which disappeared **(**□**)** and appeared **(**O**)** are indicated. The arrows indicate proteins which appeared in Figure **c** and disappeared in Figure **d**.

compared to the protein profiles of the cells harvested in early, mid and late stationary phase. This would give an indication of the number of proteins common to the various stages of the stationary phase and to the long-term starvation. Cells were subjected to multiple starvations by inoculating cells harvested in early stationary phase into sterile distilled water microcosms which should be devoid of all nutrients and hence be seen as a severe starvation medium.

Compared to the protein profiles in Figures 3a and 3b, 38 new proteins were induced during the transition from early stationary phase to starvation for 7. Eight proteins were shown to have disappeared from the protein profiles of early stationary phase cells during the 7-day starvation period. Compared to the protein profiles for mid stationary phase, cells showed that 24 new proteins were induced and 22 proteins disappeared during this stage of the starvation period (Figs.3c and 3d). The Comparison of protein profiles for the late stationary phase cells and starved cells showed that 23 new proteins could be recognized while 19 proteins disappeared during the starvation period (Figs. 3e and 3f).

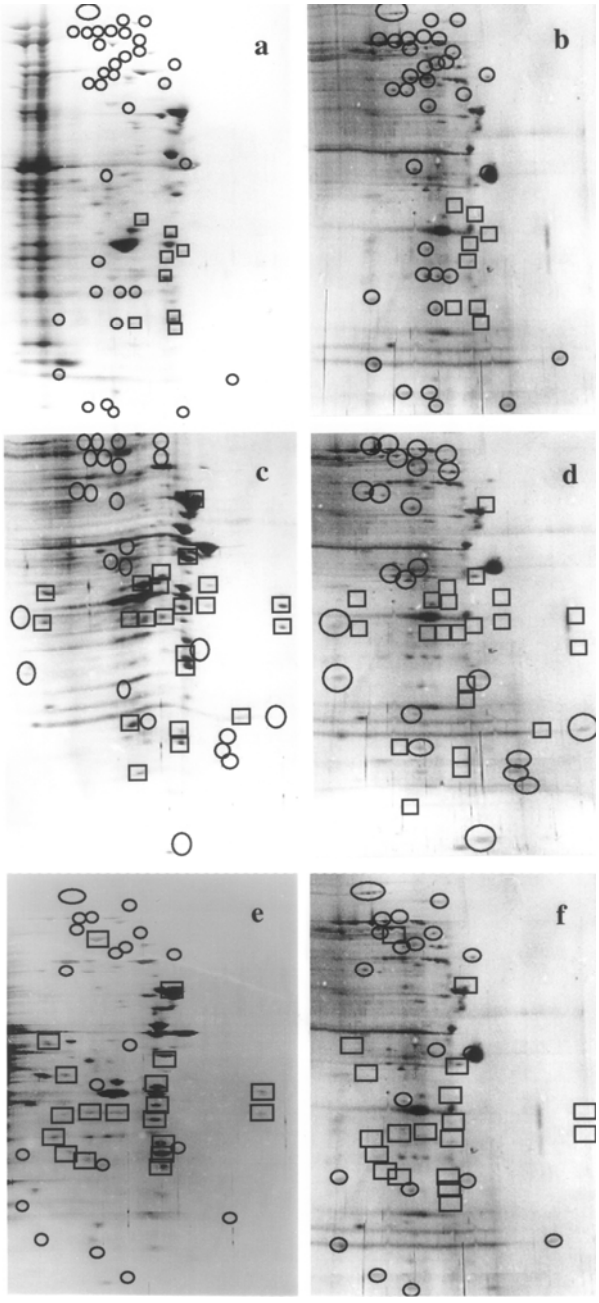


Fig. 3. Two-dimensional electrophoresis analysis. The Figure shows the protein profile **(a)** for early exponential phase cells, **(c)** for mid stationary phase cells, **(e)** for late stationary phase cells and **(b, d** and **f)** for cells subjected to multiple nutrient starvation for 7 days. Protein profiles were compared and proteins which disappeared **(**□**)** and appeared **(**O**)** are indicated.

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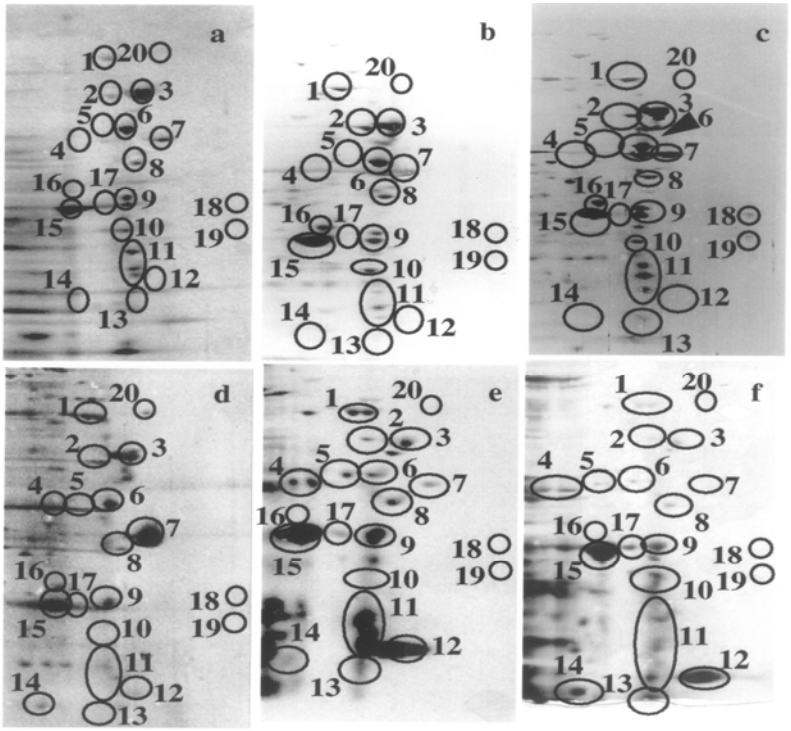


Fig. 4. Two-dimensional electrophoresis analysis. The Figure shows the protein profile **(a)** for cells harvested in the exponential phase, **(b)** cells harvested in early stationary phase, **(c)** cells harvested in late stationary phase, **(d)** cells starved for 7 days, **(e)** cells starved for 14 days and **(f)** cells starved for 28 days. The proteins which show the major changes are numbered from 1-20.

Comparison of the proteins profiles of F. chinensis under carbon, nitrogen, phosphate and multiple nutrient starvation at 15°C. Nutrients, especially carbon, nitrogen and phosphate sources, have very important roles on bacterial growth and the absence of one, or two, or all of these nutrients which have different effect on bacterial growth and, consequently, on the protein profiles produced by these cells. The effect of carbon starvation is very important as many of the proteins which are produced under carbon starvation have different roles in response to different starvation stresses, and, even, to other forms of stress. Here, the protein profiles of *F. chinensis* incubated under conditions of individual carbon, nitrogen, or phosphate limitation were compared to those of seen in non-starved bacteria harvested in the early stationary phase. The protein profiles of cells subjected to multiple nutrient starvations are shown in Figure 3. In this experiment, the cells were grown in nutrient broth until the beginning of the stationary phase of growth. The washed cells were transferred into a minimal medium from which the carbon, nitrogen and phosphate source were in turn eliminated to made a starvation medium deficient in a single nutrient source. The flasks were incubated for 7 days and the protein compared with those of cells harvested in the early stationary phase.

The comparison of the changes in protein profiles between cells of F. chinensis in the stationary phase and subjected to long-term starvation. Here, the changes in the protein profiles of cells in various stages of growth and in cells subjected to long-term starvation in sterile distilled water at 15°C were compared. Unlike the other experiments reported, each protein which showed a change was given an identifying number. Figure 4 shows the protein profiles obtained for cells harvested in the exponential phase, early stationary phase, and late stationary phase when compared with the protein profiles for cells starved for 7 days, 14 days and 28 days. Most of the new proteins which are synthesized in response to starvation appeared during the early stationary phase than in any other phase. This shows the importance of the new proteins produced in this phase of growth to the maintenance of the cell during the onset of starvation. The continued presence of these proteins in cells subjected to long-term starvation shows that the proteins have a continued role in the survival of the organisms under extreme nutrient limitation.

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The micro N-terminal sequencing of a starvation stress protein. Several starvation stress and heat shock proteins were selected as suitable for further study and transferred from the two-dimensional polyacrylamide gels into filter membrane for N-terminal micro-sequencing. Some of the proteins prepared in this way were not concentrated enough for accurate sequencing. However, one of the selected proteins was enough for sequencing. This protein was produced in response to multiple nutrient starvation and is marked in Figure 3b. The amino acid sequence was compared with the other sequences in the database using the GCG computer program. The result of the search is demonstrated in Figure 5. There was a 62.5% identity in 8 amino acid overlapped with the 5' residue of a 10-kDa chaperon protein which is known to be a protein involved in the starvation stress response in other organisms.

DISCUSSION

This study has shown that there are many proteins synthesized in common for many stresses in *F. chinensis*. Some of these proteins must play a major role in the stability of the cell under starvation stress.

The new proteins synthesized in mid and late stationary phase compared to early stationary phase showed that 12 out of 29 newly synthesized proteins from cells in the early to mid stationary phase were found in the late stationary phase cells, and 17 out of 29 new proteins were transiently induced. Their production was repressed during the transition from mid to late stationary phase and instead 15 new proteins were induced during this transition. A few proteins indicated by arrows in Figure 2c are specific to cells in the mid stationary phase and had disappeared again by the time the cells were in late stationary phase. It is assumed that these proteins are necessary for the cell to enter the starvation stage but are not essential once the cell has entered a period of prolonged starvation.

Some of the proteins as being synthesized during the transition from the early stationary phase to multiple nutrient starvations were also common to later stages of the stationary phase. This shows the link between the response to total nutrient deprivation and the inability of cells to grow in a nutrient medium during the stationary phase. It also suggests that the induction and repression of protein synthesis in these cells are much more complicated than expected. Although the starved cells have no metabolisable nutrient source, they are apparently synthesizing new proteins in response to this starvation stress to at least the same extent as cells in the stationary phase of growth.

The comparison of Figures 6a and 6b shows that 51 new proteins were induced during carbon starvation. This was the highest number of protein changes seen in response to starvation and other stresses during the course of these studies. This result demonstrated the importance of carbon sources to bacterial growth and metabolism.

The comparison of Figures 6 c and 6d shows that 31 new proteins were induced in response to nitrogen starvation. Comparison of Figures 6e and 6f shows that phosphate starvation led to the synthesis or appearance of 19 proteins.

Multiple nutrient starvations showed that 38 new proteins were induced compared to early stationary phase (Figs. 3a and 3b). Some of the proteins induced by multiple nutrient starvations were not produced under conditions of carbon, nitrogen or phosphate starvation. This again demonstrated the complicated mechanisms by which bacterial cells respond to starvation conditions.

Investigation into the appearance and disappearance of different major proteins identified on the gels showed differences at different stages of starvation. The proteins numbered 3, 6, 8, 9, and 15 were found in all the Figures. The proteins numbered 4 and 5 appeared from early stationary phase and then persisted until the end of the starvation period. The protein numbered 16 appeared in early stationary phase and was stable until 7 days of starvation but disappeared afterwards. The proteins numbered 1, 2, and 7 were found from the exponential phase until 14 days starvation but disappeared before 28 days of starvation.

The proteins numbered 12 and 17 appeared after 14 days of starvation and were then stable until the end of the starvation period. The proteins numbered 13 and 14 were only present after 7 days of starvation. The proteins numbered 18 and 19 were only present in late stationary phase cells 18 and 19 (but were also found under carbon and nitrogen limitation). The protein numbered 20 was only present in cells starved for 7 days. All these results show that changes in the protein profiles are occurring constantly even though these cells are subjected to extreme starvation conditions.

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Sw:ch10_bruab
ID   CH10_BRUAB   STANDARD;      PRT;    98 AA
AC   P255968;
DE   10 KD CHAPERONIN (PROTEIN CPN 10) (PROTEIN GROES)….
62.5% identity in 8aa overlap
X              MRTIQFRPHI
               |    |   | | |
Ch10_b         MADIKFRPLHDRVVVRRVESEAKTAGGIIIPDTAKEKPQEGE
               1            10             20            30            40


Sw:ch11_rhime
ID   CH11_RHIME   STANDARD;      PRT;    98 AA
AC   P35472;
DE   10 KD CHAPERONIN  A (PROTEIN CPN10  A) (PROTEIN GROES A)….
50.0%  identity in 8 aa overlap
X              MRTIQFRPHI
               |         | | |
Ch11_r         MASTNFRPLHDRVVVRRVESEEKTKGGIIIPDTAKEKPQEGE
               1            10             20            30            40


Sw:ch12_braja
ID   CH12_BRAJA    STANDARD;      PRT;    104 AA
AC   P35863;
DE   10 KD CHAPERONIN  2 (PROTEIN CPN10  2) (PROTEIN GROES 2)….
60.0% identity in 5aa overlap
X    MRTIQFRPHI

               | | |
ch12_b   MKFRPLHDRVVVKRIDAEEKTAGGIIIPDTVKEKPSQGEVIAVGPGG
         1             10             20            30            40


Sw:ch13_braja
ID   CH13_BRAJA    STANDARD;      PRT;    104 AA
AC   P35864;
DE   10 KD CHAPERONIN  3 (PROTEIN CPN10  3) (PROTEIN GROES 3)….
60.0% identity in 5aa overlap
X    MRTIQFRPHI
               | | |
ch13_    MKFRPLHDRVVVKRIDAEEKTAGGIIIPDTVKEKPSQGEVIAVGPGG
         1             10             20            30            40


Sw:ch13_rhime
ID   CH11_RHIME   STANDARD;      PRT;    101 AA
AC   P35474;
DE   10 KD CHAPERONIN  C (PROTEIN CPN10  C) (PROTEIN GROES C)….
60.0% identity in 8aa overlap
X    MRTIQFRPHI
               | | |
ch13_r   MAFRPLHDRILVRRVESEEKTKGGIIIPDTAKEKPQEGEEVLAVGPG
         1             10             20            30            40
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Fig. 5. The micro N-terminal sequence and homology identity of a starvation protein.

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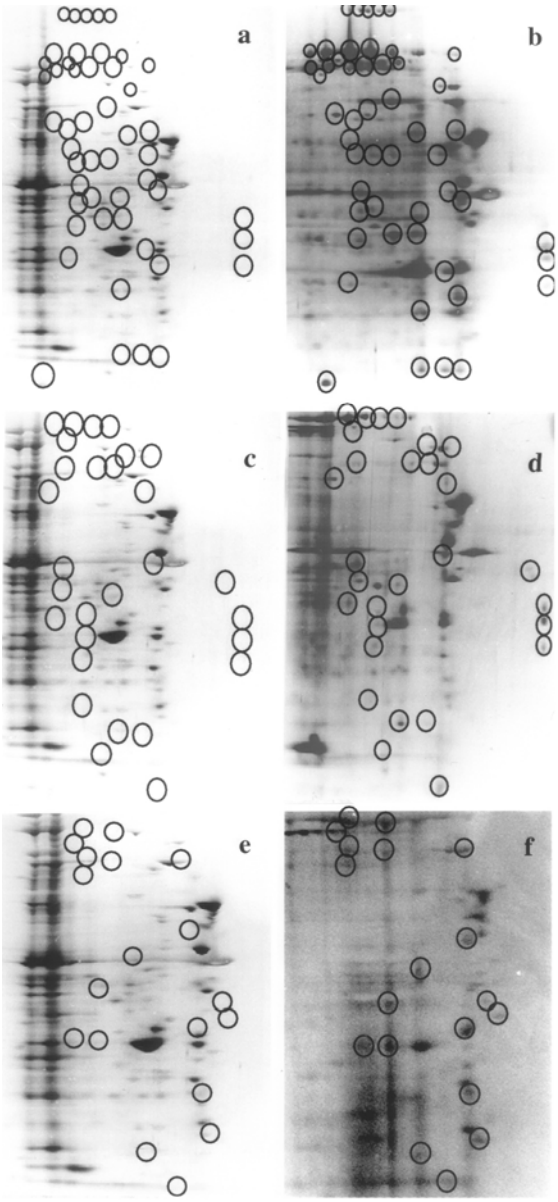


Fig. 6. Two-dimensional electrophoresis analysis. The Figure shows the protein profile (**a**, **c** and **e**) for early exponential phase cells, **(b)** for cells starved for carbon, **(d)** for cells starved for nitrogen and **(f)** for cells starved for phosphate for 7 days. All samples were at 15°C. Protein profiles were compared and proteins which disappeared (□) and appeared (O) are indicated.

Kramer and Singleton [14] reported that in *Vibrio furnissii* in response to nutrient depletion, cell mass and rRNA decreased rapidly while the total cell counts remained stable. This reduction in cell mass is seen as miniaturization of cells, similar to the phenomenon observed in other starved *Vibrio* species. The overall rate of protein synthesis in *Vibrio S14* after 24 h of starvation was about 10% of that of cells growing exponentially in a complex medium and the global rate of protein synthesis reduced by 95% in *E. coli* [15]. In both cases the proteins which were being synthesized were the ones specifically associated this starvation stress and the development of resistance to stress in these bacteria. The rate of total protein synthesis decreased immediately at the onset of starvation and after 48 h was less than 1% of the rate of synthesis during growth [16].

In the experiments reported here, the total number of proteins seen in profiles of *F. chinensis* run on one-dimensional polyacrylamide gels showed several changes over a 7-day period of starvation.

During the transition on the stationary phase at temperatures around the optimum for growth, the concentration of proteins which are involved in transcription and translation is greatly reduced. However, some proteins which have roles in energy metabolism are greatly increased in *E. coli* [17]. To eliminate some of the changes in protein profiles in starvation medium which could be induced by high temperature, all the experiments in starvation medium were conducted at 15°C. This is the normal temperature range for growth of *F. chinensis* but is less than the optimum temperature for growth of this organism, to obtain only the starvation proteins in absences of the effect of high temperature.

Reeve *et al.* [18] reported that, in *E. coli*, protein synthesis during the first 9 h of starvation was the most crucial for survival and the hypothesis that these proteins play a role in long-term starvation is supported by the observation that the addition of chloramphenicol or amino acid at the beginning of the starvation period had a negative effect on culture viability.

Nystrom [1] reported that carbon depletion is the determinant for several events including survival stress resistance and changes in the kinetics of proteins and RNA synthesis which also occur under multiple nutrient starvations. In these experiments, carbon starvation did not appear to be the major determinant for protein expression during multiple nutrient starvations. The carbon starvation stimulon did not appear to overlap with the multiple nutrient starvation stimulon to greater extent that did the nitrogen and phosphate starvation stimulon. A small number of proteins belonging to the carbon, nitrogen and phosphate starvation-stimulons were induced regardless of the starvation condition employed. Some of the phosphate starvation-specific proteins were not induced during simultaneous starvation for

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phosphate, nitrogen and carbon. Finally, a large number of proteins were induced exclusively in response to multiple nutrient starvations and could not be grouped into any of the individual nutrient starvation stimulons. This suggests that the multiple nutrient starvation stimulon is not only the sum of individual-nutrient starvation stimulons, and that, probably, additional sensors or signals are involved in the response to simultaneous starvation for carbon, nitrogen and phosphorus as has also been suggested by Nystrom [1].

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