

“Research Note”

**THE EFFECT OF STARVATION FACTOR ON THE SURVIVAL
CHARACTERISTICS OF FLEXIBACTER CHINENSIS***

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Abstract – To survive in natural waters, bacteria must respond to a variety of environmental variables such as starvation. *Flexibacter chinensis* was grown and multiplied in the starvation medium and its viable count, total count and cell size were investigated under different temperatures. Also, the effects of different nutrient sources were investigated. The survival of the organism under starvation conditions was temperature dependent with the longest survival occurring at 4°C and the shortest at above 30°C. Amendments of starvation medium with glucose or urea (as carbon and nitrogen source respectively) delayed the reduction in cell size and increased the survival time of *F. chinensis* at a number of different temperatures.

Keywords – *Flexibacter chinensis*, stress, starvation

1. INTRODUCTION

The *Flexibacter* genus belongs to the flexibacter-flavobacter-cytophaga group of organisms. The genus is gram negative, chemoorganotroph, nonphotosynthetic, and facultative anaerobes. Some of this genus species have pathogenic importance (in aquatic animals), while others have pharmacologic importance [1].

One of the remarkable features of bacterial species is their capacity for rapid growth when the essential nutrient sources and appropriate conditions are available for growth [2]. If one or several of these essential nutrients become limiting and fall below the threshold concentrations, the cell will become growth-arrested [3]. Temperature can also be an important factor in determining whether or not a cell can enter into a dormant or viable but non-culturable state [4].

In this study, the effects of the starvation factor and different nutrient sources on the survival of *Flexibacter chinensis* have been investigated. The effects of this factor on cell size, and viable and total counts over a long term starvation period have been quantified.

2. MATERIALS AND METHODS

a) Bacterial Strain

Bacterial Strain *Flexibacter chinensis* was obtained from Ken Flint, Warwick University (UK).

b) Bacterial Growth Media

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The bacteria strain was routinely grown in Luria Broth (LB) (10g/l Bacto tryptone, 5g/l yeast extract, 5g/l NaCl, pH 7.2) or on Luria Agar (LBA) (10g/l bacto tryptone, 5g/l yeast extract, 5g/l NaCl, 15g/l Agar).

c) Starvation protocol

F. chinensis was grown in Luria broth at 30°C for 24 hr (180 rpm). The Culture (10 ml) was centrifuged and washed twice using sterile distilled water and the pellet resuspended in a 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). Viable counts were determined on LBA plates after 48 hr incubation at 30°C.

d) Viable count

The viable count was determined using a surface spread plate technique. Samples (1 ml) were taken from the flasks and serial dilutions prepared in Quarter-strength Ringers solution (2.25g/l NaCl, 0.12 g/l CaCl_2 , 0.05 g/l NaHPO_4 and 0.105 g/l KCl in 1 liter Distilled water). Plates were counted by using an illuminated colony counter. The results were expressed as cfu/ml.

e) Total Counts

The total count was determined using a Coulter counter ZM (Coulter Euro Diagnostics (GMBH) with a 30 μm orifice probe. The data were analyzed using Coulter channelyzer software to estimate the size distribution. This software also determines mean cell size and volume.

f) Nutrient Source Amendments

To investigate the response of the cell to individual nutrient starvation, a minimal medium, 100 mM Tris buffer PH:7.4 (500 ml), 1 gr/l CaCl_2 (100 ml), 10gr/l $(\text{NH}_4)_2\text{SO}_4$ (100ml), 10 gr/l $\text{MgSO}_4.7\text{H}_2\text{O}$ (10 ml), 0.005gr/l $\text{FeSO}_4.7\text{H}_2\text{O}$ (10ml), 2.5ml trace element (including 232 mg $(\text{NH}_4)_2\text{FeSO}_4$, 464 mg H_3BO_3 , 191 mg CuSO_4 , 348 mg $\text{ZnSO}_4. 7\text{H}_2\text{O}$ in 1000ml distilled water) and 77.5 ml distilled water were used [1]. D-glucose (as a Carbon source) with 0.5 g/l, KH_2PO_4 (as a phosphorus source) and urea (as a nitrogen source) with 100 mg/ml were used.

3. RESULTS

a) The effect of temperature and starvation stress on *Flexibacter chinensis*

The survival of *F. chinensis* in a starvation medium at different temperatures at a range from 4°C to 37°C was compared. Figure 1 shows that the reduction in the viable count of *F. chinensis* in the starvation medium was dependent upon the incubation time and temperature. At 37°C, there were no viable cells detectable by plate counts after 2 days incubation. The viable count decreased faster at 30°C than at the other temperatures. At 4°C and 15°C, viability was relatively constant for the first 24 days of the starvation period. There was a small increase in the total cell numbers during the first 12-18 days starvation at all temperatures (Fig. 2). This increase was most apparent at 15°C (the optimum growth temperature for this bacterium), while the smallest increase was at 4°C. While none of the increases in the total cell count was significant, they were reproducible in all of the starvation experiments conducted in this study. The cell size of *F. chinensis* decreased during the 60 day starvation period (Fig. 3). The reduction in cell size was dependent upon the incubation temperature, with the smallest reduction occurring at 4°C and the largest reduction at 30°C.

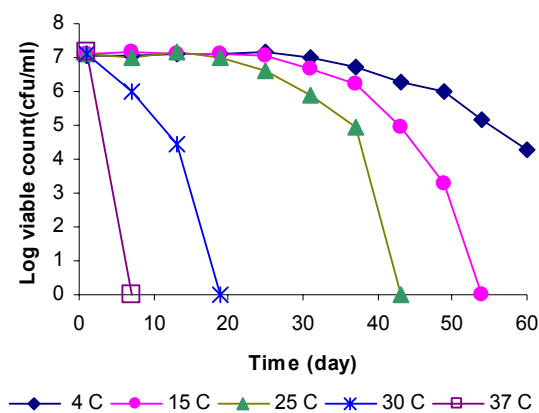


Fig. 1. Viable count variation. The effect of different temperatures on the viable count of *F. chinensis* in a starvation medium. In all figures microcosms (100 ml) were inoculated to give an initial viable of 10^7 cfu/ml

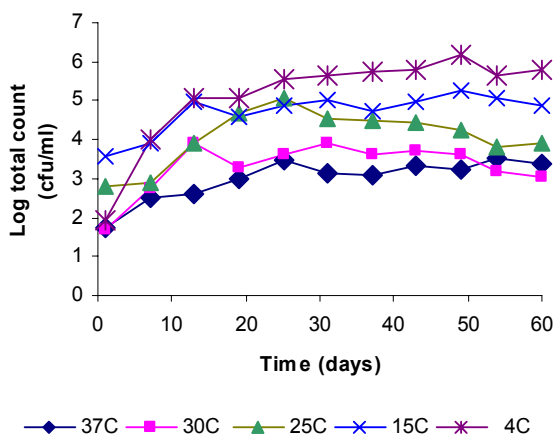


Fig. 2. Total count variation. The effect of different temperatures on the total cell count of *F. chinensis* in a starvation medium

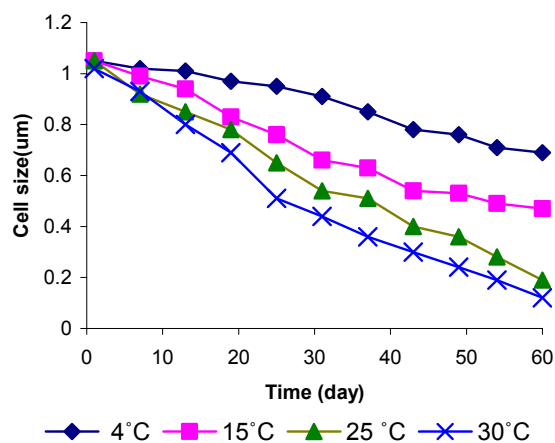


Fig. 3. Cell size variation The reduction in cell size in *F. chinensis* in a starvation medium. Cell size is equivalent to spherical diameters

b) The effect of nutrient sources on the survival of *F. chinensis* in a starvation medium at 15°C

In this experiment, the effect of different nutrient amendments was examined to find the relation between the nutrient amendment and the survival of *F. chinensis*. The addition of D-glucose as a carbon source led to an increase in the retention of cell viability in comparison to the unamended control (Fig. 4). The time taken for a two-log decrease in the viable cell count was increased from 36 days to 46 days after the addition of D-glucose. There was no significant increase in the total cell count. Cell size also decreased more slowly in the amended microcosms, again suggesting that there was a link between cell survival and decrease in cell size.

Urea was used as a nitrogen source amendment. Again the amendment increased the time taken for a two-log decrease in the viable count from 36 days to 51 days (Fig. 5). And the viable count was detectable for 68 days in the amended samples compared to 54 days in the unamended control. The increase in survival was more enhanced after the addition of urea than after the addition of D-glucose.

The addition of Potassium dihydrogen orthophosphate as an amendment had no effect on the viable or total cell counts, or the cell size of *F. chinensis* when compared with the unamended control (Data not shown).

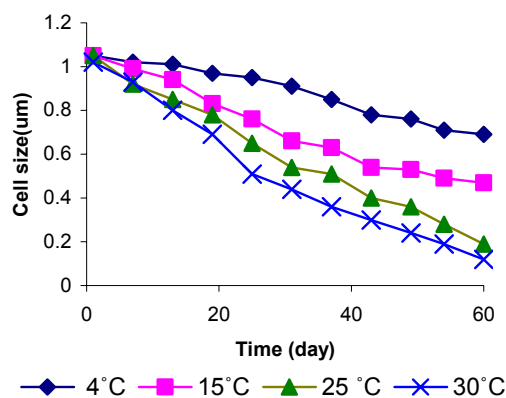


Fig. 4. Effect of D-glucose amendment on total count, viable count and cell size of *F. chinensis* at 15°C

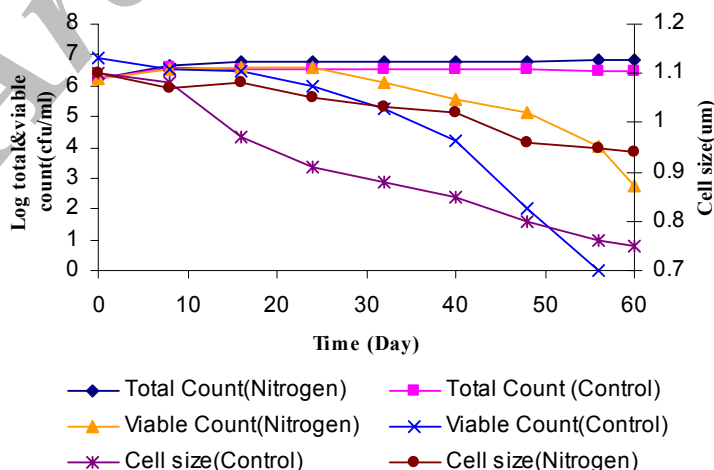


Fig 5. Effect of Urea amendment on total count, viable count and cell size of *F. chinensis* at 15°C

4. DISCUSSION

Using sterile distilled water as a severe form of starvation medium for *F. chinensis*, survival times were compared at different temperatures. The longest survival time was at 4 °C and the lowest at 30°C. Above 30 °C, viability was very quickly lost and no viable cells could be detected at 37°C. Ozkanca [5] reported that *E. coli* cells survived for a longer period of time in sterile river water at 4°C than at 37°C and viability was not lost rapidly until the incubation temperature of the starvation medium was increased above 45°C.

In the experiments reported in this study, the total count remained relatively constant at all temperatures, however, usually a small but not significant increase in the total count was occasionally at 4°C. The results suggest that, in this starvation medium, the best temperature for *F. chinensis* cell growth is 15°C. Although the total counts for an over 60 day starvation period, the cell viable counts decreased with the rate of decrease dependent upon the temperature of incubation. The fact that total counts remain constant, although there is a decrease in the viable count, suggests that cells are not lysing, and therefore it is considered more likely that the cells have become viable but non-culturable cells rather than dead cells.

Comparing Figs. 1, 2 and 3 shows that there is a relationship between the decrease in viable count and the cell size reduction. There was a greater decrease in the viable count at 30°C, and this was matched by a greater decrease in cell size. It is interesting to note that the decrease in cell size is almost linear at temperatures. At 30 °C, after 18 days starvation, the viable cell count had become undetectable but cell size continued to decrease until the end of the starvation experiment at 60 days.

The addition of D-glucose as a carbon source and urea as a nitrogen source to the starvation medium at 15°C led to both an increase in cell numbers and the survival time of *F. chinensis*. A similar result was reported by Lim [6] using *A. hydrophila*. It is, perhaps, significant that both *F. chinensis* and *A. hydrophila* are typical aquatic bacteria and can respond more positively to an added carbon and nitrogen source in a dilute medium than can a more fastidious organism such as *E. coli*.

The amendment of the microcosms with a nitrogen source led to a higher increase in survival times than using carbon or phosphate as amendments.

The addition of an inorganic phosphate to the starvation medium had no effect on the survival or on the cell numbers of *F. chinensis*.

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