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The Effect of Some Environmental Factors on the Survival Characteristics of *Flexibacter chinensis*

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Abstract

To survive in natural waters, bacteria must respond to variety of environmental factors. *Flexibacter* survival in the aquatic environment is influenced by number of parameters, such as temperature, heavy metal concentration and oxidation. Here we investigate the influence of these factors on the survival of *Flexibacter chinensis* in starvation medium. Our data shows that different concentrations of hydrogen peroxide decrease the survival time of bacteria while they have no influence on total count. Also, the investigation about the effect of heat shock on this bacterium demonstrates that the increasing of temperature leads to viability decrease. At least, the examination of the effect of heavy metal ions on the survival of *F. chinensis* shows that Zinc had less inhibitor activity on the cell viability in comparison to Cadmium and Copper.

Keywords: *Flexibacter chinensis*; Stress

Introduction

The Flexibacter genus belongs to the flexibacter-flavobacter-cytophaga group of organisms. The genus is gram negative, chemoorganotroph, nonphotosynthetic, facultative anaerobes and its habitat is fresh water and soil [1].

Some of this genus species have pathogenic importance; special inhibitors, such as novel inhibitors of mammalian DNA Topoisomerase-1 [2], and an inhibitor of human leukocyte elastase are produced by some species of flexibacter [3]. Also, there are some pathogenic species of flexibacter which produce disease, especially in fish culturing which are of economic importance [4].

Generally, prokaryotic cells respond to environmental or chemical stresses by inducing specific sets of proteins characteristic of each stress [5]. In some cases, such as sub lethal concentration of hydrogen peroxide (a side product of oxygen metabolism), these proteins cause metabolic changes [6]. Fridovich [7] suggested that every organism which uses molecular oxygen has to protect itself against the toxic effects of hydrogen peroxide. Catalase is one of the enzymes which are involved in the degradation of hydrogen peroxide, and its production is increased in response to a sub-lethal concentration of hydrogen peroxide [8].

The heat shock response is one of the best studied stress responses in bacteria and is responsible for the increased heat resistance in bacteria exposed to elevated temperatures. In *E. coli*, cell survival is increased at elevated temperatures dependent upon the temperature

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of pre-incubation of the cells even in the absence of the nutrients [9]. However, high temperature also causes several forms of injury in bacterial cells, at even a few degrees above the maximum growth temperature [10]. A sudden increase in temperature results in transient induction of a small set of proteins concomitant with the transient shut down of the synthesis of many constitutive proteins [11]. In response to carbon starvation in *E. coli*, more than 30 proteins are induced which are necessary for starvation survival. Among these proteins are other stress-related protein, including 11 heat shock proteins, 6 oxidative stress proteins and 5 osmotic stress proteins. Similarly several properties, which are not produced during exponential growth, are exhibited in stationary- phase bacterial cultures. These properties include increased proteolysis, the induction of about 40 starvation-related proteins, and enhanced resistance to heat, oxidative stress, and antibiotics [12].

In this study, the effects of a variety of shocks (including hydrogen peroxide, high temperature and different concentration of heavy metals), on the survival of *F. chinensis* have been investigated. The effects of these factors on cell size, viable and total counts over a long term starvation period have been quantified.

Materials and Methods

Bacterial Strain

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

Bacterial Growth Media

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

Heat Shock

F. chinensis was grown in LB for 24 h at 30°C. Cells (10 ml) were centrifuged (5000 g, 10 min), and washed twice in sterile distilled water and 0.1 ml of the resuspended cells were inoculated into 100 ml of sterile water in a 250 ml flask to give an initial viable count of approximately 10^7 cfu/ml. The flask was kept in the range of 30°C to 55°C for the required length of time [13].

Hydrogen Peroxide Shock

Flasks were prepared as mentioned above. However, instead of being placed at 55°C, the flasks were amended by the addition of hydrogen peroxide to a final concentration of 18 mM (621 mg/ml) at 15°C [13].

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 (2.25 g/*l* NaCl, 0.12 g/*l* $CaCl_2$, 0.05 g/*l* $NaHPO_4$ and 0.105 g/*l* KCl in one liter Distilled water). The viable counts were determined on Luria Bertani agar plates after incubation at 30°C for 48 h. Plates were counted by using an illuminated colony counter. The results were expressed as cfu/ml [14].

Total Counts

Samples were diluted in an isotonic buffer containing 0.4% (v/v) glutaraldehyde to fix the cells. 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. The software also determines mean cell size and volume [13,15].

Electron Transport System Assay (INT Method)

In order to investigate the viable but non-culturable cell the activity of the Electron Transport System (ETS) was measured using the method of Prin *et al.* [16]. Samples (10 ml) were added to 20 ml sterile plastic universals wrapped in foil. 0.1 ml of 0.2% (w/v) INT (2, 9-p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) was added and the universal was incubated at 25°C for 24 h without shaking. INT activity was stopped by the addition of 0.1 ml of 37% (v/v) formaldehyde. The cells were pelleted by centrifugation at 13800 g for 10 min at 4°C. The supernatant was poured off and 1 ml methanol was added for 2 h to extract the INT-formazan from the cells. The cell debris was removed by centrifugation, and the absorbance was read at 490 nm using methanol as a blank.

Heavy Metals Shock

F. chinensis was grown overnight at 30°C in LB. The cells were harvested, washed and resuspended in sterile water. Sterile water microcosms (100 ml) were inoculated to give an initial viable count of 10^7 cfu/ml, and were amended with different concentration of copper, zinc, and cadmium to give a final concentration

of the heavy metal ion of 1, 5, 10 and 20 ppm. The flasks were incubated at 15ºC [13]. For recovery, bacteria were grown in LB for 48 h at 30°C. After this step, the cells were recovered and again became culturable and viable.

Results

The Effect of Hydrogen Peroxide on the Survival of F. chinensis

F. chinensis was exposed to hydrogen peroxide (3 and 10 mM final concentration) in sterile distilled water as a starvation medium (data not shown). When a concentration of 10 mM hydrogen peroxide was used, the viable count decreased to an undetectable level between 2 days and 4 days after incubation at 15°C. The total count did not decrease suggesting that cells do not get lysed under starvation condition but become non-culturable. Cells exposed to a final concentration of hydrogen peroxide of 3 mM survived for up to 18 days but this was still less than the survival time of the control cells. There was a small but not significant increase in the total count in the control flask but no increase in total count numbers in either of the amended flasks. There was no viable count detectable in flasks amended with 18 mM hydrogen peroxide after 2 days.

Any attempt to recover the cells, exposed to the effect of 18 mM hydrogen peroxide, in different recovery media, such as diluted nutrient broth, failed. Starvation of the cells in sterile distilled water at 15°C for 30 days before exposure to 18 mM hydrogen peroxide resulted in the production of a resistant population of cells. After the starved cells had been exposed to 18 mM hydrogen peroxide, recovery was attempted by incubating the starved cells in 1:5 diluted nutrient broth. Figure 1 shows that viable cells were detected after 2 days and the viable count was increased to almost the same value as the total count after 6 days incubation at 15°C. The respiration activity of these cells showed a similar recovery (Fig. 1). There was no detection of respiration activity or recovery of cell viability non-starved cells treated in exactly the same manner.

The Effect of Heat Shock on the Survival of F. chinensis

The effect of temperature in the range from 30°C to 55°C on the survival of *F. chinensis* in sterile distilled water was examined. No viable count was detectable at 55°C after 6 h, but the cells retained viability for up to 12 h at 45°C. At 37°C and 30°C, the cells remained culturable for 2 days and 8 days, respectively (Fig. 2).

As in the experiment with bacteria, starved cells which had been incubated at 55°C for 24 h were recovered after the starvation medium was transferred to 15°C. In the case of the recovery from oxidative shock the cells required a dilute nutrient broth medium to aid recovery, here the cells recovered viability and respiration activity in the sterile distilled water medium. It is interesting to note that respiration activity was detectable at day 0 before the cells became culturable after 2 days (Fig. 3).

The Effect of Heavy Metal Ions on the Survival of Flexibacter chinensis

Cadmium, copper and zinc, at a range of concentrations from 1 *ppm* to 20 *ppm*, were used to study the effects of heavy metal ions on the survival of *F. chinensis* in sterile distilled water microcosms. The control samples were an inoculation of bacteria in sterile distilled water without any additional metal ions. Zinc (Fig. 4a) had less inhibitor activity on cell viability than cadmium (Fig. 4b) or copper (Fig. 4c). With the addition of zinc, the effect on cell viability was greatest at 20 *ppm* zinc amendment, but even 1 *ppm*, reduced the time for a one log decrease in viability from in excess of 15 days to 10 days. Copper and cadmium had a very similar effect on the viable count of *F. chinensis*. There was no detectable viable count after the addition of either ion at 20 *ppm* within 24 h and at a concentration of 10 *ppm*, there was no detectable viable count after 2 days. A comparison of the effects of the same concentration of ion shows that the time for a one log decrease in viability is 3 days for copper, 4 days for cadmium and 10 days for zinc.

Discussion

Subjecting cells to some shocks gave an indication of the cross- relatedness of the response to stress in bacterial cells. Hydrogen peroxide probably has lethal effect in *F. chinensis* at a concentration of 18 mM in sterile distilled water. Cell culturability by viable count was completely lost in non-starved cells subjected to the addition of 18mM hydrogen peroxide. Any attempt to recover these stressed cells in different minimal or diluted nutrient broth media failed. However, starved cells of *F. chinensis* were more resistant to the lethal effects of hydrogen peroxide. Despite the absence of growth on nutrient agar plates after exposure to hydrogen peroxide these cells were recoverable in a dilute nutrient broth medium.

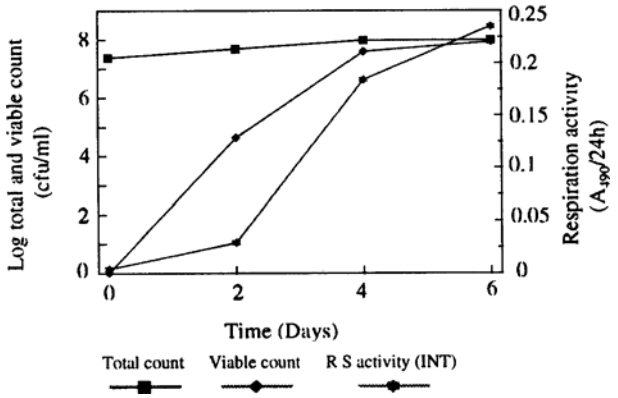


Figure 1. Recovery of starved *F. chinensis* after exposure to hydrogen peroxide.

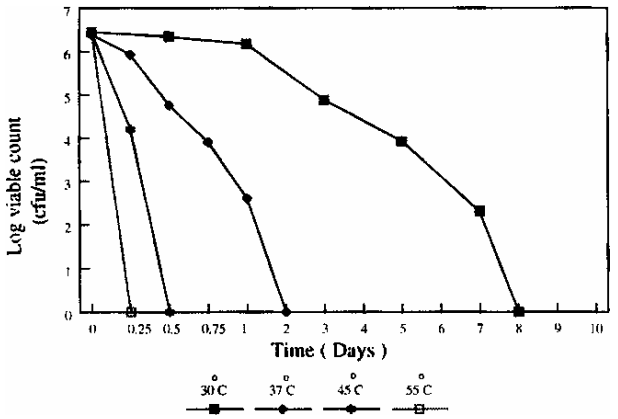


Figure 2. The effect of heat shock on the survival of *F. chinensis*.

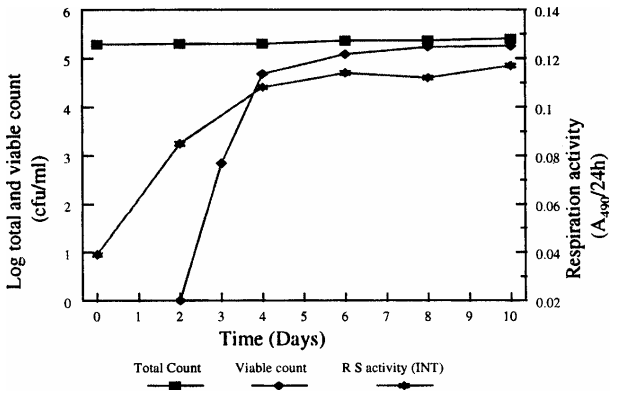
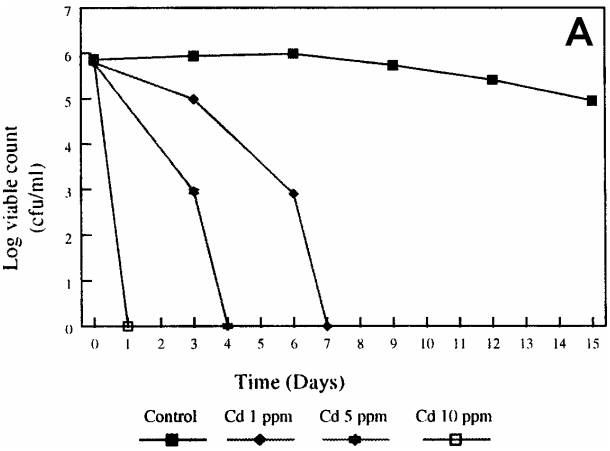
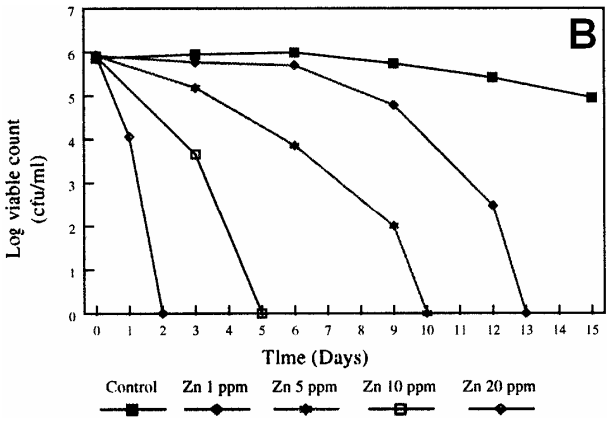


Figure 3. The recovery of heat shocked *F. chinensis* in a starvation medium.





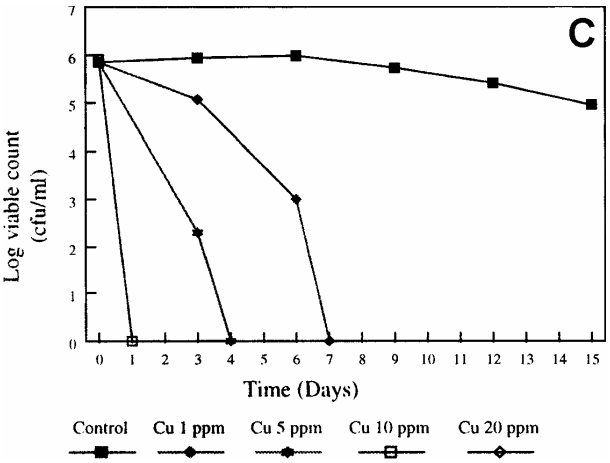


Figure 4. The effects of heavy metal ions on the viable count of *F. chinensis* under starvation conditions.

Exposure of *E. coli* cells to hydrogen peroxide at low concentrations prior to the introduction of the cells to starvation medium enhanced the survival still further in the presence of higher concentrations of hydrogen peroxide [17]. It has been suggested that the increased survival could have been due to the induction of catalase by exposure to low levels of hydrogen peroxide but equally this enhanced resistance could be due to changes in the cellular physiology due to starvation. Christman *et al.* [18] reported that *E. coli* under hydrogen peroxide stress produced many novel proteins. Some of them also enhanced the ability of the bacteria to survive under starvation stress [19]. Ozkanca [20] reported that, although pre-treatment of *E. coli* with hydrogen peroxide resulted in a loss of viability in the first few days of starvation, after recovery, there was a similar increase in bacterial numbers back to original inoculum size. It is unlikely that this is simply due to the growth of a single resistant cell as there was no increase in the total cell count. Lim and flint [21] also showed a similar ability of *E. coli* to recover completely from the effects of heat stress in a recovery medium. In their case, the temperature of recovery was important but this was not investigated further in this study.

Similarly here, *F. chinensis* completely lost culture-ability within a couple of hours when subjected to heat shock at 55°C. However, at 37°C, viability was lost after two days incubation. This is a more rapid loss of culturability than previously shown for *E. coli* [21], but this is a more heat sensitive organism having an optimum growth temperature of 15°C [16]. The cells which had been exposed to 55°C recovered in diluted nutrient broth after two days of incubation at 15°C (in this case respiration activity was detectable from the start of the recovery period). Moats [22] reported that *E. coli* could recover its full viability after heat shock, given a supply of nutrients and the right storage conditions. *F. chinensis* also recover from oxidative shock given the right incubation and recovery conditions.

The effect of heavy metal ions on the survival of *F. chinensis* was examined. Lim [23] reported that heavy metal ions could alter the metabolism or morphology of a cell or affect the survival of a bacterium. Here, zinc had less toxicity than copper or cadmium at the same concentration with the complete loss of culturability occurring at 10 *ppm* final concentration of both copper and cadmium within 24 h of exposure. Lim [23] showed that 5 *ppm* of copper led to complete loss of culturability of *E. coli* within 24 h when the bacteria were subjected to metal stress under starvation conditions in lake water. As with *F. chinensis*, some culturability of *E. coli* was maintained at all concentrations of zinc up to 20 *ppm*.

References

1. Bergey D.H., Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T., and Williams S.T. *Bergey's Manual of Determinative Bacteriology*. 9th Edition, Williams & Wilkins, Baltimore, USA, 483-514 pp. (1994).
2. Suzuki K., Yamaguchi H., Miazaki S., Nagai K., Watanabe S., Saito T., Ishaii K., Hanada M., Seklen T., Ikegami Y., and Andoh T. Topostine, a novel inhibitor of mammalian and topoisomerase-1 from Flexibacter topostinus, sp. nov. 1. taxonomy, and fermentation of producing strain. *Journal of Antibiotics*, **43**: 154-157 (1990).
3. Fujita T., Okamato M., Kosako Y., and Okuhara M. *Flexibacter japonensis* SP-NOV a new species that produces a novel inhibitor of human leukocyte elastase isolated from soil. *Current Microbiology*, **33**: 89-93 (1996).
4. Strunjak-perovic I., Hacmanjek M., Coz-Rakoovac R., Teskeredzic E., Teskeredzic Z., and Topic-Papovic N. Bacterial disease in sea fish. *Ribarstov*, **55**: 147-160 (1995).
5. Jenkins D.E., Schultz J.E., and Matin A. Starvation induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. *Journal of Bacteriology*, **170**: 3910-3914 (1988).
6. Farr S.B. and Kogoma T. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiological Reviews*, **55**: 561-585 (1991).
7. Fridovich I. The biology of the oxygen radicals. *Science*, **201**: 375-880 (1978).
8. Fridovich I. Free radicals. In: W.A. Pryor (Ed.), *Biology*, Academic Press, New York, 239-277 pp. (1976).
9. Tsuchido T., Takano M., and Shibasaki I. Effect of temperature elevating process on the subsequent isothermal death of *Escherichia coli*. *Journal of Bacteriology*, **131**: 848-853 (1974).
10. Allwood M.C. and Russell A.D. Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool in *Staphylococcus aureus*. *Ibid.*, **95**: 345-349 (1968).
11. Spencer J., Cegielska A., and Georgopoulos C. Role of *Escherichia coli* heat shock proteins DnaK and HtpG (C62.5) in response to nutritional deprivation. *Ibid.*, **172**: 7157-7166 (1990).
12. Jenkins D.E., Auger E.A., and Marin A. Starvation-induced cross protection against osmotic challenge in *Escherichia coli*. *Ibid.*, **172**: 2779-2781 (1990).
13. Raheb J. The molecular and physiological effects of starvation and other stresses on *Flexibacter chinesis*. *Ph.D. Thesis*, University of Warwick, U.K. (1998).
14. Ozkanca R. and Flint K.P. Alkaline phosphatase activity of *Escherichia coli* starved in sterile lake water

microcosms. *Journal of Applied Bcteriology*, **80**: 252-258 (1996).

15. Ovez B., Mergaret J., and Saglam M., Biological denitrification in drinking water treatment using the seaweed *Gracilaria verrucosa* as carbon source and biofilm carrier. *Water Environment Research*, **78**: 430-434 (2006).
16. Prin Y., Neyra M., and Diem H.G. Estimation of *Frankia* growth using Bradford protein and INT reduction activity estimations: application to inoculum standardization. *FEMS Microbiology and Ecology*, **69**: 91-96 (1990).
17. Demple B. and Halbrook J. Inducible repair of oxidative damage in *Escherichia coli*. *Nature*, **304**: 466-468 (1983).
18. Christian M.F., Morgan R.W., Jacobson F.S., and Ames B.N. Positive control of a regulon for defense against oxidative stress and some heat shock proteins in *Salmonella typhimurium. Cell*, **41**: 753-762 (1985).
19. Reeve C.A., Amy P.S., and Matin A. Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *Journal of Bacteriology*, **160**: 1041-1046 (1984).
20. Ozkanca R. Survival and physiological of *Escherichia coli* in lake water under different nutrient conditions. *Ph.D. Thesis*, University of Warwick, U.K. (1993).
21. Lim C.H. and Flint K.P. The recovery of heat-stressed *Escherichia coli* in lake water microcosoms. *Letters in Applied Microbiology*, **21**: 364-367 (1995).
22. Moats W.A. Kinetics of thermal death of bacteria. *Journal of Bacteriology*, **105**: 161-171 (1971).
23. Lim C.H. Laboratory studies of the survival of *Escherichia coli* in lake water. *M.Sc. Thesis*, University of Warwick, U.K. (1988).